

# Promiscuous targeting of polytopic membrane proteins to SecYEG or YidC by the *Escherichia coli* signal recognition particle

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**ABSTRACT** Protein insertion into the bacterial inner membrane is facilitated by SecYEG or YidC. Although SecYEG most likely constitutes the major integration site, small membrane proteins have been shown to integrate via YidC. We show that YidC can also integrate multispanning membrane proteins such as mannitol permease or TatC, which had been considered to be exclusively integrated by SecYEG. Only SecA-dependent multispanning membrane proteins strictly require SecYEG for integration, which suggests that SecA can only interact with the SecYEG translocon, but not with the YidC insertase. Targeting of multispanning membrane proteins to YidC is mediated by signal recognition particle (SRP), and we show by site-directed cross-linking that the C-terminus of YidC is in contact with SRP, the SRP receptor, and ribosomal proteins. These findings indicate that SRP recognizes membrane proteins independent of the downstream integration site and that many membrane proteins can probably use either SecYEG or YidC for integration. Because protein synthesis is much slower than protein transport, the use of YidC as an additional integration site for multispanning membrane proteins may prevent a situation in which the majority of SecYEG complexes are occupied by translating ribosomes during cotranslational insertion, impeding the translocation of secretory proteins.

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## INTRODUCTION

Transport of proteins from the cytoplasm into the cytoplasmic membrane is an essential process in bacterial physiology. To facilitate protein insertion into lipid membranes, efficient transport systems have evolved, which include the membrane-embedded

SecYEG translocon and the YidC insertase (Pohlschröder *et al.*, 2005; Driessen and Nouwen, 2008). The SecYEG translocon is the major protein-conducting channel in the bacterial cytoplasmic membrane. It is involved in both membrane protein insertion and in secreting proteins across the membrane into the periplasm (Rapoport, 2007).

The transport of periplasmic proteins across the SecYEG translocon proceeds in a posttranslational manner and depends on ATP hydrolysis by the SecYEG-associated motor protein SecA. In contrast, inner membrane proteins (IMPs) need to be inserted into the membrane cotranslationally as ribosome-nascent chain complexes (RNCs). Cotranslational targeting and insertion is mediated by the signal recognition particle (SRP), which recognizes an emerging signal anchor sequence (Neumann-Haefelin *et al.*, 2000; Bornemann *et al.*, 2008) and targets RNCs to the membrane-bound SRP receptor FtsY. FtsY occupies the ribosome-binding site of SecY (Kuhn *et al.*, 2011) and is probably displaced by the SRP-RNCs, which ensures efficient docking of the RNCs onto the SecYEG translocon. The hydrophobic transmembrane (TM) helices that emerge from the ribosomal tunnel exit are inserted into the SecY channel and

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Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; IMP, inner membrane protein; INV, inverted inner membrane vesicle; KOAc, potassium acetate; Mg(OAc)<sub>2</sub>, magnesium acetate; MPF, membrane protected fragment; MtIA, mannitol permease; pBpa, para-benzoyl-L-phenylalanine; PK, proteinase K; pmf, proton-motive force; RNC, ribosome-associated nascent chain; SRP, signal recognition particle; TeaOAc, triethanolamine acetate; TM, transmembrane.

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further transferred into the lipid bilayer. The latter step is probably facilitated by a lateral opening of SecY (Van den Berg *et al.*, 2004). SecA has also been found to cooperate with the SecYEG translocon for the translocation of large periplasmic domains of inner membrane proteins (Deitermann *et al.*, 2005). In addition, SecYEG associates with other membrane-embedded components such as YidC (Scotti *et al.*, 2000) to mediate the insertion of TMs. In vitro cross-linking studies using ribosome-nascent chains of IMPs such as manitol permease (MtlA) or FtsQ have shown length-dependent contacts of TMs to SecY, YidC, and lipids (Beck *et al.*, 2001; van der Laan *et al.*, 2001). It was therefore suggested that YidC is located at the lateral gate of SecY, where it supports the partitioning of TMs into the lipid bilayer. In addition, YidC has been suggested to participate in the folding (Nagamori *et al.*, 2004), assembly (Wagner *et al.*, 2008), and quality control of membrane proteins (van Bloois *et al.*, 2008).

YidC belongs to an evolutionarily conserved family of proteins, which include the mitochondrial homologues Oxa1 and Cox18 and the chloroplast homologues Alb3 and Alb4 (Funes *et al.*, 2011). All members of the YidC protein family have a conserved core of five TMs that in *Escherichia coli* and other Gram-negative bacteria is connected via a long periplasmic loop to an additional N-terminal TM (Sääf *et al.*, 1998; Ravaud *et al.*, 2008). Other functional additions to the conserved core were acquired during evolution (Funes *et al.*, 2011), such as a C-terminal extension in some YidC variants of Gram-positive bacteria, Alb3, and Oxa1 (Funes *et al.*, 2009). This extension was shown to serve as a ribosome-binding site (Szyrach *et al.*, 2003). YidC is essential in *E. coli* but can be functionally replaced by the mitochondrial Oxa1 (van Bloois *et al.*, 2005). A complementation of Oxa1 by YidC is, however, only possible when the C-terminal ribosome-binding site of Oxa1 is attached to YidC (Preuss *et al.*, 2005). Depletion of YidC in *E. coli* results in a global change in cell physiology (Price *et al.*, 2010; Wickström *et al.*, 2011), where chaperones such as Trigger Factor and FimC are up-regulated and stress-responsive pathways like Cpx are induced (Wang *et al.* 2010).

In addition to its SecYEG-associated function during membrane protein insertion, YidC can insert membrane proteins independent of SecYEG. YidC was shown to insert small proteins such as phage proteins (Samuelson *et al.*, 2000; Serek *et al.*, 2004; Klenner *et al.*, 2008). YidC was also shown to insert the F<sub>0</sub>C subunit of the F<sub>0</sub>F<sub>1</sub>-ATP synthase (van der Laan *et al.* 2003, 2004a; Yi *et al.*, 2003) and MscL (Facey *et al.*, 2007), although the exact role of YidC in MscL integration is controversial (Pop *et al.*, 2009; Berrier *et al.*, 2011).

So far the substrates described as being inserted via YidC are small, closely spaced membrane proteins with short hydrophilic loops. How these proteins are targeted to YidC and why they evade the SecYEG translocon are unclear. A possible involvement of SRP has been proposed for the targeting of F<sub>0</sub>C (van der Laan *et al.*, 2004a; van Bloois *et al.*, 2004) and MscL (Facey *et al.*, 2007; Pop *et al.*, 2009), but a direct interaction of YidC with components of the SRP pathway has not been detected in bacteria. In contrast, the chloroplast Alb3 has been shown to interact with FtsY (Moore *et al.*, 2003) and with cpSRP43, which replaces the 4.5S RNA in the chloroplast SRP (Falk *et al.*, 2010; Richter *et al.*, 2010). A recent cryo-electron microscopic (cryo-EM) study of a YidC-RNC complex indicated that the ribosome contacts YidC via the ribosomal proteins L23, L24, and L29 (Kohler *et al.*, 2009). Because these proteins are located close to the ribosomal tunnel exit and are also involved in SecY binding (Frauenfeld *et al.*, 2011), it was suggested that the ribosome has common binding sites for both the SecYEG translocon and the YidC insertase.

In the present study, we address the substrate specificity of YidC and demonstrate that YidC also inserts multispansing membrane proteins in vitro, which had been considered to be exclusively inserted via SecYEG. Only SecA-dependent membrane proteins cannot be inserted via YidC. Targeting to YidC is mediated by SRP, and site-directed cross-linking shows that the short C-terminus of YidC is in contact with the ribosome, SRP, and FtsY. These in vitro data suggest that the SRP pathway delivers membrane proteins cotranslationally to two distinct integration sites at the *E. coli* membrane.

## RESULTS

### Functional reconstitution of SecYEG and YidC into proteoliposomes

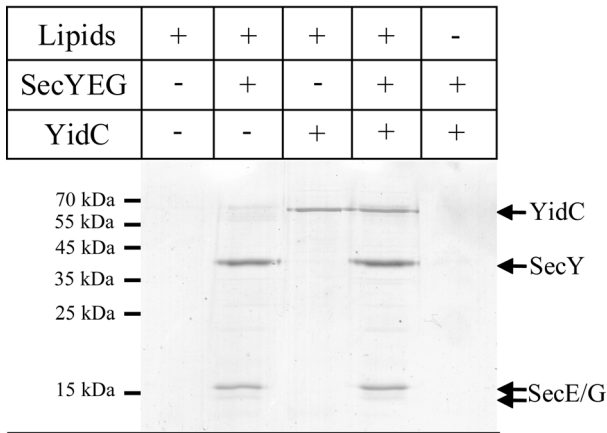
Identifying the determinants that route proteins into the YidC-only or into the SecYEG/YidC pathway by in vivo experiments has been complicated because the conditional depletion of SecY or YidC induces a multifaceted response that includes the up-regulation of proteases and chaperones (Baars *et al.*, 2008; Price *et al.*, 2010; Wang *et al.*, 2010; Wickström *et al.*, 2011). In addition, it has been impossible to generate an *E. coli* strain in which SecY and YidC can be inactivated individually or together (Pop *et al.*, 2009). Finally, as the integration of YidC is SecY dependent (Koch *et al.*, 2002) and the integration of SecY is probably influenced by YidC depletion (Price *et al.*, 2010), experiments using the available depletion strains are extremely challenging. We therefore chose to address this question in vitro by using reconstituted proteoliposomes. Purified YidC and SecYEG were reconstituted into liposomes containing native *E. coli* lipids supplemented with 5% diacylglycerol (DAG), which has been shown to reduce nonphysiological spontaneous membrane protein insertion (Nishiyama *et al.*, 2006). The proteoliposomes were then isolated by centrifugation and separated on SDS-PAGE. Coomassie blue staining revealed no protein contaminants in the pure liposome fraction (Figure 1A), whereas in SecYEG proteoliposomes, SecY and SecE were detectable. Coomassie blue only weakly stains SecG (Nishiyama *et al.*, 2006), which explains why it was barely visible on the gel (Figure 1A). In YidC proteoliposomes, YidC was detected as a 60-kDa band, and in proteoliposomes coreconstituted with SecYEG and YidC, all proteins except SecG were detectable in comparable amounts. In the absence of lipids, neither SecYEG nor YidC was found in the pellet fraction after centrifugation, indicating that they did not aggregate. To exclude that the 60- to 70-kDa protein contaminants in purified SecYEG (Figure 1A) corresponded to YidC, we performed Western blotting, which revealed that SecYEG proteoliposomes did not contain detectable amounts of YidC. We also showed that YidC proteoliposomes were free of SecY (Figure 1B).

The purified in vitro transcription/translation system (TT system) used in this study does not contain significant amounts of FtsY, Ffh, SecA, SecY, or YidC (Koch *et al.*, 1999; Figure 1C). Thus combining an in vitro system free of membranes and targeting factors with reconstituted proteoliposomes allowed us to determine the requirements for membrane protein integration in *E. coli*.

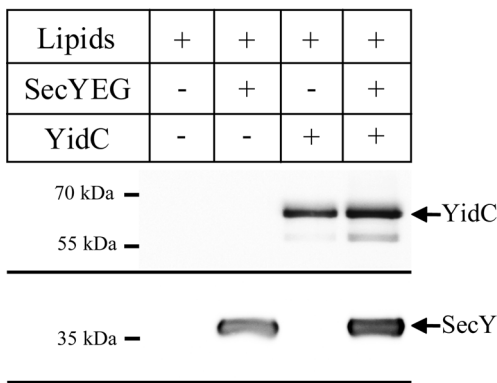
### The polytopic membrane protein TatC is targeted by SRP to either SecYEG or YidC

As a first substrate, we chose an N-terminally histidine (His)-tagged version of TatC, a multispansing membrane protein with a predicted molecular mass of 29 kDa. With its predicted six closely spaced TMs (Behrendt *et al.*, 2004; Figure 2A), TatC is more complex than the so-far-identified substrates of the YidC-only pathway. When TatC was synthesized in vitro in the presence of *E. coli* inner membrane vesicles (INVs), we observed a membrane-protected fragment (MPF)

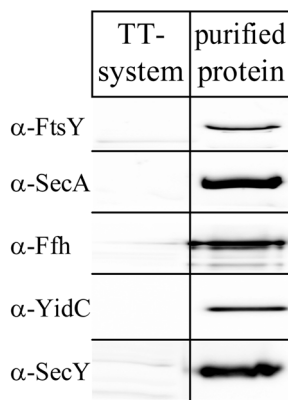
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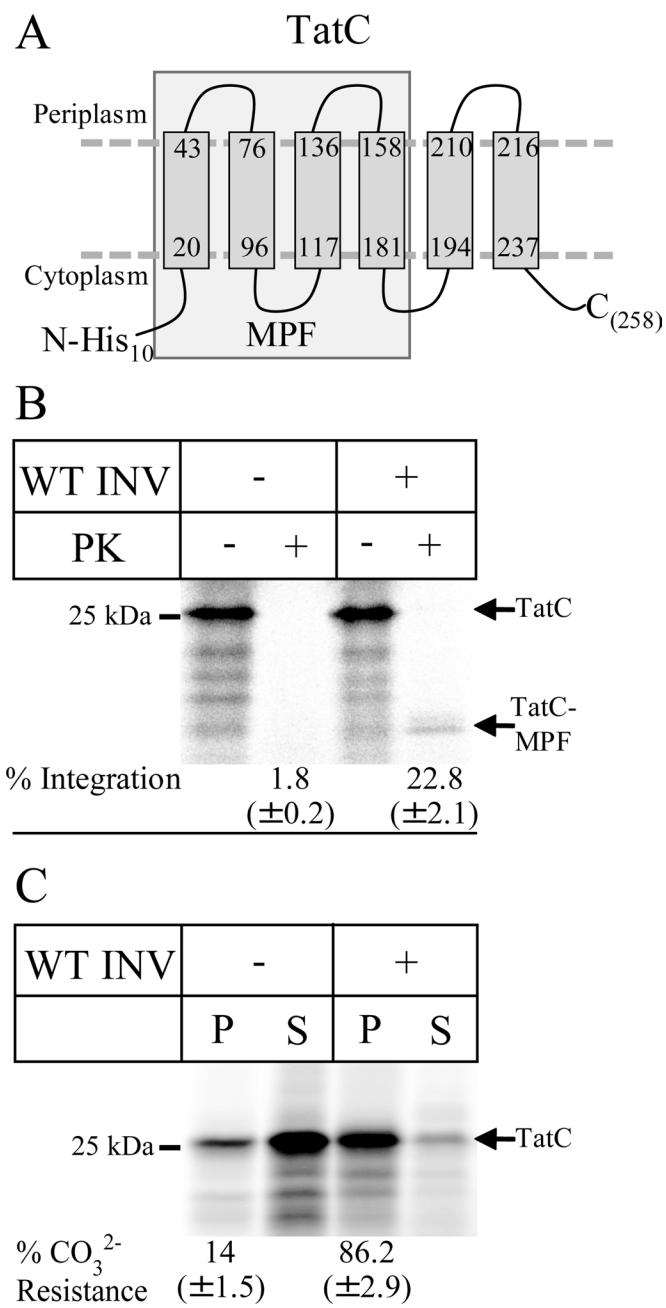


**FIGURE 1:** Reconstitution of SecYEG and YidC into proteoliposomes. (A) Proteoliposomes were prepared using *E. coli* phospholipids supplemented with 5% DAG. Purified SecYEG or YidC were reconstituted into liposomes and pelleted by centrifugation. The pellet was resuspended and adjusted to a final concentration of 0.4 mg/ml SecY, 0.4 mg/ml YidC, or 0.4 mg/ml SecY + 0.4 mg/ml YidC. One aliquot (4  $\mu$ l) was loaded onto a 15% SDS gel and Coomassie stained. Pure liposomes served as control. (B) The proteoliposomes (2  $\mu$ l) shown in A were separated on SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was subsequently cut into two pieces, and the upper part was decorated with  $\alpha$ -YidC antibodies and the lower part with  $\alpha$ -SecY antibodies. (C) One aliquot of the purified *E. coli* in vitro TT system used for in vitro protein synthesis was probed with the indicated antibodies after Western blotting. Purified FtsY (0.7  $\mu$ g), Ffh (0.2  $\mu$ g), SecA (1.5  $\mu$ g), SecY (0.8  $\mu$ g), and YidC (0.8  $\mu$ g) served as controls.

of ~19 kDa after proteinase K (PK) treatment (Figure 2B) that was not observed in the absence of INVs. A similar protease-protected fragment was also observed in in vivo pulse-chase experiments (Yi *et al.*, 2003), but the exact nature of the MPF is unknown. We therefore constructed two truncated TatC derivatives, which lacked either the first two TMs (TatC $\Delta$ N) or the last two TMs (TatC $\Delta$ C). The migration of TatC $\Delta$ C on SDS-PAGE was identical to the migration of TatC-MPF, whereas TatC $\Delta$ N migrated faster than the TatC-MPF (Supplemental Figure S1). This indicates that the MPF probably corresponds to the first four TMs of TatC. However, due to the irregular migration behavior of some membrane proteins on SDS-PAGE, we cannot entirely exclude that the TatC-MPF corresponds to all six TMs. The membrane integration of TatC into *E. coli* INVs was further confirmed by alkaline carbonate extraction, a method that is routinely used to differentiate between membrane-inserted and soluble proteins (Fujiki *et al.*, 1982). In the presence of INVs, almost 90% of TatC was found in the pellet fraction after carbonate extraction and centrifugation, indicating membrane insertion (Figure 2C). Carbonate treatment of in vitro-synthesized TatC in the absence of INVs resulted in >80% of TatC in the supernatant (Figure 2C). Differences in the integration rates of TatC measured by carbonate extraction or PK digestion are likely due to additional PK cleavage sites resulting in smaller PK resistant fragments, which are not included in calculating the integration rate.

When TatC was synthesized in the presence of liposomes, the 19-kDa MPF was not detectable (Figure 3A). Instead, we noticed some protease-resistant, full-size TatC, which probably reflects TatC aggregates. In the presence of SecYEG proteoliposomes, the MPF of TatC was reproducibly detected when SRP and the bacterial SRP receptor FtsY were present (Figure 3A), but the total integration was significantly lower than in INVs (Figure 2B). Only a weak protease protection of TatC was observed in the absence of SRP/FtsY. We next analyzed whether coreconstituting YidC and SecYEG into proteoliposomes would increase the efficiency of TatC integration as would be expected if YidC and SecYEG cooperated during the integration of membrane proteins. Protease protection of TatC in the presence of SRP/FtsY was significantly stronger in SecYEG/YidC proteoliposomes than in SecYEG proteoliposomes (Figure 3A) supporting a cooperative function of YidC with SecYEG in membrane integration. We also noticed that although TatC integration into SecYEG/YidC proteoliposomes was most efficient when both SRP and FtsY were present, significant integration was also observed in their absence (Figure 3A). Owing to the intrinsic affinity of SecYEG for ribosomes (Prinz *et al.*, 2000), a weak SRP/FtsY-independent integration is expected and was observed for other SecYEG-dependent membrane proteins in proteoliposome studies (Braig *et al.*, 2011). Because the presence of YidC seemed to increase the SRP/FtsY-independent integration of TatC, we analyzed proteoliposomes reconstituted with YidC alone. It is striking that we observed that TatC was more efficiently integrated into YidC proteoliposomes than into SecYEG-proteoliposomes (Figure 3A). Integration of TatC via YidC was most efficient in the presence of SRP/FtsY but also occurred in the presence of SRP alone.

The integration of TatC into liposomes and proteoliposomes was further analyzed by carbonate extraction, and these assays were performed in the presence of SRP/FtsY. In the presence of liposomes, only ~10% of in vitro-synthesized TatC was found in the pellet fraction (Figure 3B), which was comparable to the values observed in the absence of membranes (Figure 2C). We observed only a weak insertion of TatC into SecYEG proteoliposomes as indicated by carbonate resistance, whereas TatC was integrated very efficiently into YidC proteoliposomes (Figure 3B). Coreconstituting



**FIGURE 2:** Integration of in vitro-synthesized TatC into inner membrane vesicles. (A) Predicted topology of TatC according to Behrendt *et al.* (2004); a His<sub>10</sub> tag was fused to its N-terminus. The boxed portion of TatC most likely corresponds to the 19-kDa, membrane-protected fragment of TatC (TatC-MPF). (B) <sup>35</sup>S-labeled His<sub>10</sub>-TatC was in vitro synthesized in the absence (-INV) or presence of wild-type *E. coli* inner membrane vesicles (WT INV; 2 mg/ml). After synthesis, one-fourth of the reaction was precipitated with TCA, and the remainder was first treated with 0.5 mg/ml PK for 30 min at 25°C and then TCA precipitated. Full-size TatC (TatC) and the TatC-MPF are indicated. Note that wild-type *E. coli* INV contains sufficient amounts of SRP and FtsY (Koch *et al.*, 1999). The percentages of PK protection were calculated using ImageQuant (GE Healthcare) by quantifying the ratio of radioactivity present in the PK-treated sample and the directly TCA-precipitated sample and are the mean values of at least three independent experiments. Note that the calculation is corrected for the loss of methionine/cysteine residues and based on the assumption that TatC-MPF corresponds to the first four TMs of TatC. (C) TatC was in vitro synthesized as in B but extracted with alkaline Na<sub>2</sub>CO<sub>3</sub>

SecYEG together with YidC did not increase membrane insertion of TatC (Figure 3B). These data are consistent with the protease protection assays (Figure 3A), which also showed that TatC is inserted more efficiently into YidC proteoliposomes than into SecYEG proteoliposomes.

YidC could facilitate the integration of multispanning membrane proteins like TatC by forming a protein-conducting channel like SecY, but it could also disrupt the close spacing of the phospholipid head groups in the lipid bilayer, subsequently allowing TMs to insert at the protein-lipid interface. We verified the specificity of YidC-dependent TatC integration by constructing the YidC(I361S) mutant, in which isoleucine at position 361 in TM2 of YidC was replaced by serine. This mutation has been shown to reduce the integration of the M13-coat-Lep fusion protein, a bona fide YidC substrate (Jiang *et al.*, 2003). YidC(I361S) was purified and reconstituted into proteoliposomes in comparable amounts to YidC in YidC proteoliposomes (Figure 3D). We observed that TatC was integrated at a lower efficiency in YidC(I361S) proteoliposomes than in YidC-proteoliposomes (Figure 3D), consistent with earlier data (Jiang *et al.*, 2003). This indicates that multispanning substrates like TatC and phage proteins follow a similar insertion path through YidC.

The TatC construct used in our experiments contained an N-terminal (His)<sub>10</sub> tag and we wanted to exclude that the His tag influences the integration of TatC via YidC. We found that untagged TatC acquired protease protection like (His)<sub>10</sub>TatC in YidC proteoliposomes but not in YidC(I361S) proteoliposomes (Figure 3E), demonstrating that the (His)<sub>10</sub> tag did not influence YidC-dependent integration.

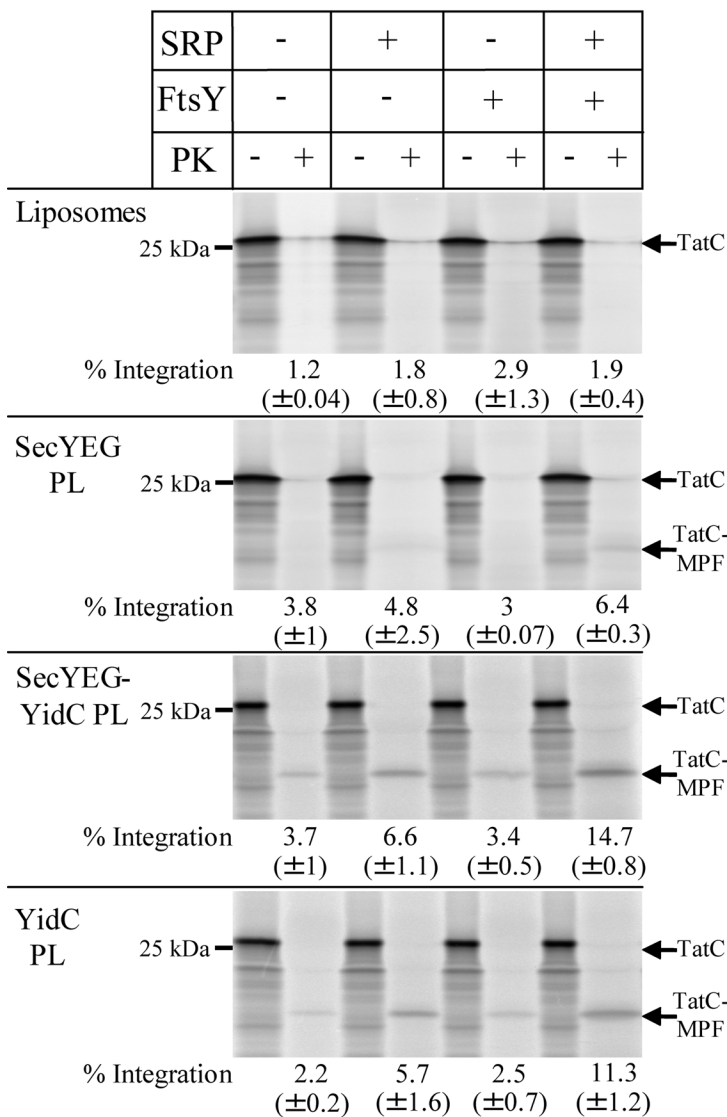
In summary, our data indicate that membrane insertion via the YidC-only pathway is not limited to small membrane proteins, but that YidC can also insert multispanning membrane proteins like TatC. Targeting of TatC to YidC is enhanced by SRP but appears to be less dependent on FtsY. TatC can also be inserted by SecYEG, but this pathway is less efficient. In SecYEG/YidC proteoliposomes, it is difficult to determine whether SecYEG and YidC function as distinct entities or whether they cooperate during TatC integration. The observation that FtsY-independent integration was predominantly observed in YidC proteoliposomes and SecYEG/YidC proteoliposomes could indicate that they function mainly independently.

#### YidC mediates integration of mannitol permease independent of the SecYEG translocon

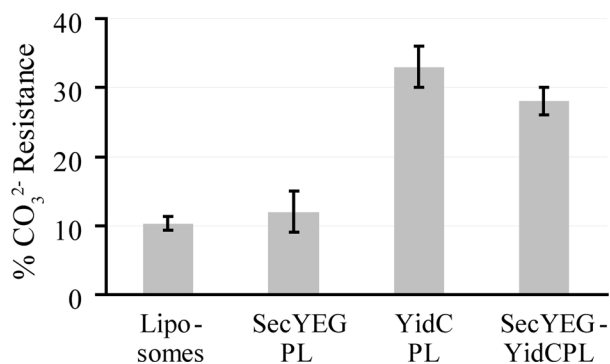
The 60-kDa membrane protein MtlA was previously used as a model substrate for studying SRP-dependent targeting to the SecYEG translocon (Koch *et al.*, 1999; Beck *et al.*, 2000; Braig *et al.*, 2011). Because we observed the insertion of TatC into YidC proteoliposomes, we also analyzed MtlA insertion into YidC proteoliposomes. Like TatC, MtlA consists of a hydrophobic core of six TMs and an additional 30-kDa cytoplasmic domain (Figure 4A). Integration of MtlA into INVs is indicated by a 30-kDa MPF after PK treatment. This product corresponds to the hydrophobic six-TM core of MtlA but lacks the cytosolic domain, which is cleaved off by PK (Werner *et al.*, 1992; Koch *et al.*, 1999; Figure 4B). The protease-sensitive band at ~32 kDa (Figure 4B, asterisk) is probably the result of premature

(pH 11.3, 0.2 M final concentration). After ultracentrifugation, pellet (P) and supernatant (S) were separated by SDS-PAGE. For quantification, the amounts of radioactive material in both fractions were set as 100%, and the distribution between both fractions was calculated. The values provided are the mean values of at least three independent experiments, and the SD is indicated.

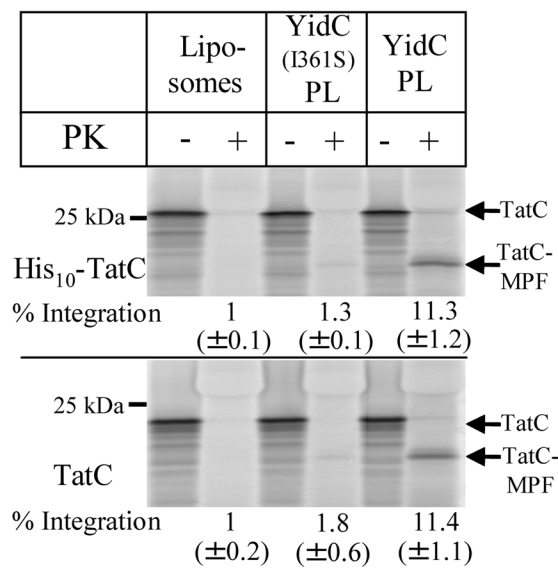
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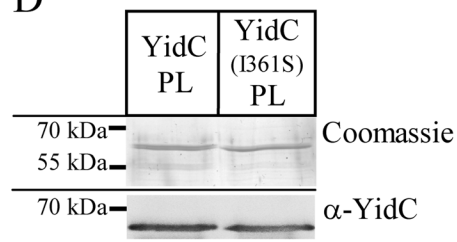
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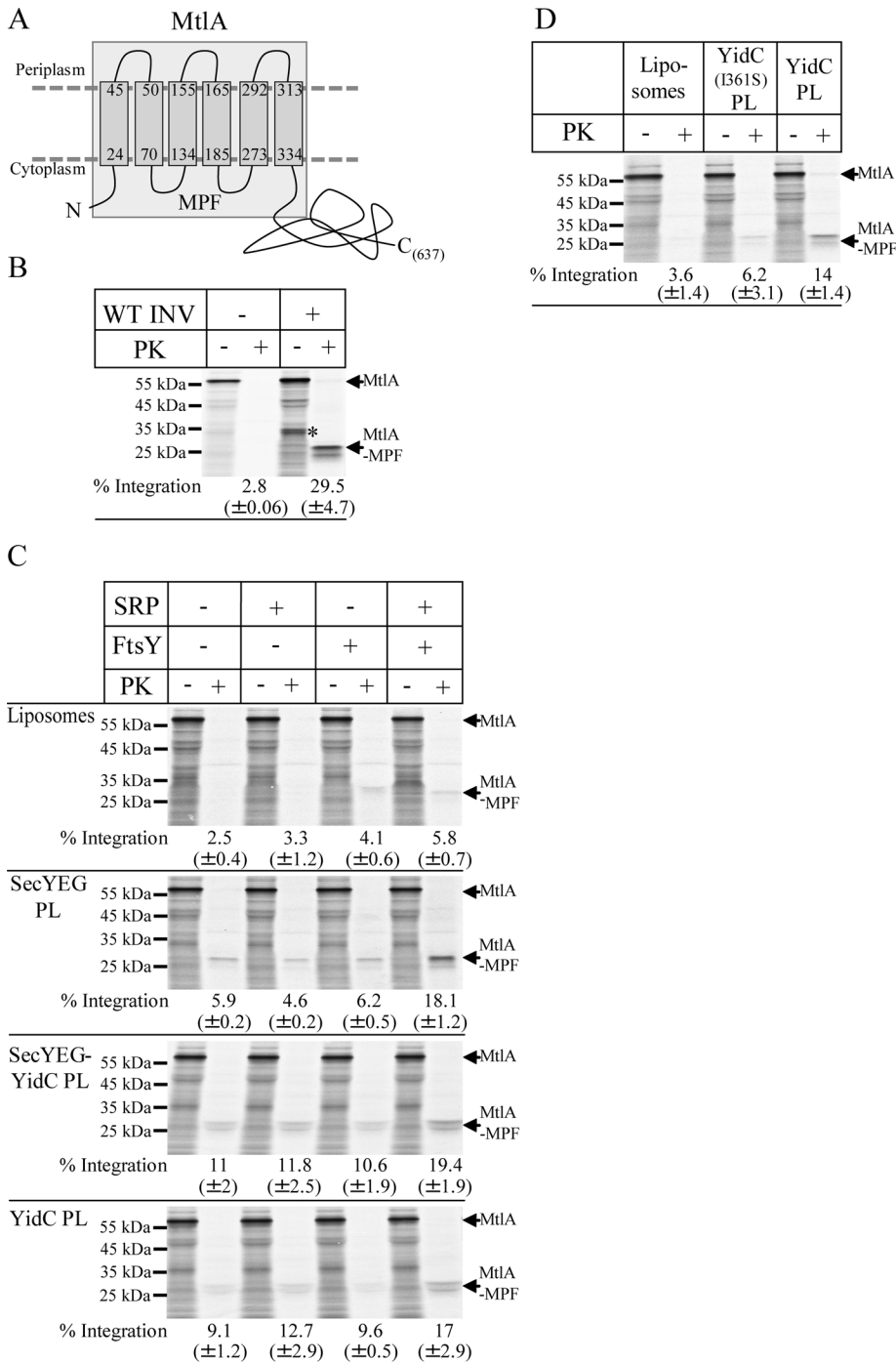
C



D



**FIGURE 3:** TatC is targeted by SRP to either SecYEG or YidC. (A) <sup>35</sup>S-Labeled His<sub>10</sub>-TatC was in vitro synthesized as described in Figure 1. When indicated, SRP (Ffh [150 nM] + 4.5S RNA [15 μg/ml]) and FtsY (750 nM) were present during synthesis. Synthesis was performed in the presence of 0.4 mg/ml of the liposomes or proteoliposomes (PL) shown in Figure 1. After synthesis, the samples were PK digested. (B) Carbonate resistance of TatC synthesized in the presence of liposomes or proteoliposomes. The mean values of three independent experiments are shown. (C) <sup>35</sup>S-Labeled His<sub>10</sub>-TatC and <sup>35</sup>S-labeled TatC were synthesized in the presence of SRP, FtsY, and proteoliposomes reconstituted with either wild-type YidC or the YidC(I361S) mutant. (D) Coomassie blue staining and immunodetection of proteoliposomes containing either wild-type YidC or the YidC(I361S) mutant. Four μl of each proteoliposome preparation were loaded for Coomassie staining and 2 μl for immunodetection.



**FIGURE 4:** YidC is sufficient for MtlA integration. (A) Topology of MtlA according to Sugiyama *et al.* (1991); the cleavage site of PK is indicated. The MPF corresponds to the first six TMs. (B) <sup>35</sup>S-Labeled MtlA was synthesized in the presence (WT INV) or absence (-INV) of INV. After synthesis, one-half of the reaction was precipitated with TCA, and the other half of the reaction was first treated with 0.5 mg/ml PK for 30 min at 25°C and then TCA precipitated. Full-length MtlA and the membrane-protected fragment (MtlA-MPF) corresponding to the hydrophobic core of MtlA are indicated. The band labeled with an asterisk probably corresponds to a product of premature termination of protein synthesis. (C) MtlA was synthesized as described in B and under the same conditions as described in the legend to Figure 3A. (D) MtlA was synthesized as described in Figure 3C.

termination of *in vitro* protein synthesis. In the presence of liposomes, we observed a weak spontaneous insertion of MtlA when SRP/FtsY was added (Figure 4C). This was observed previously and probably reflects TMs that penetrate between head groups of phos-

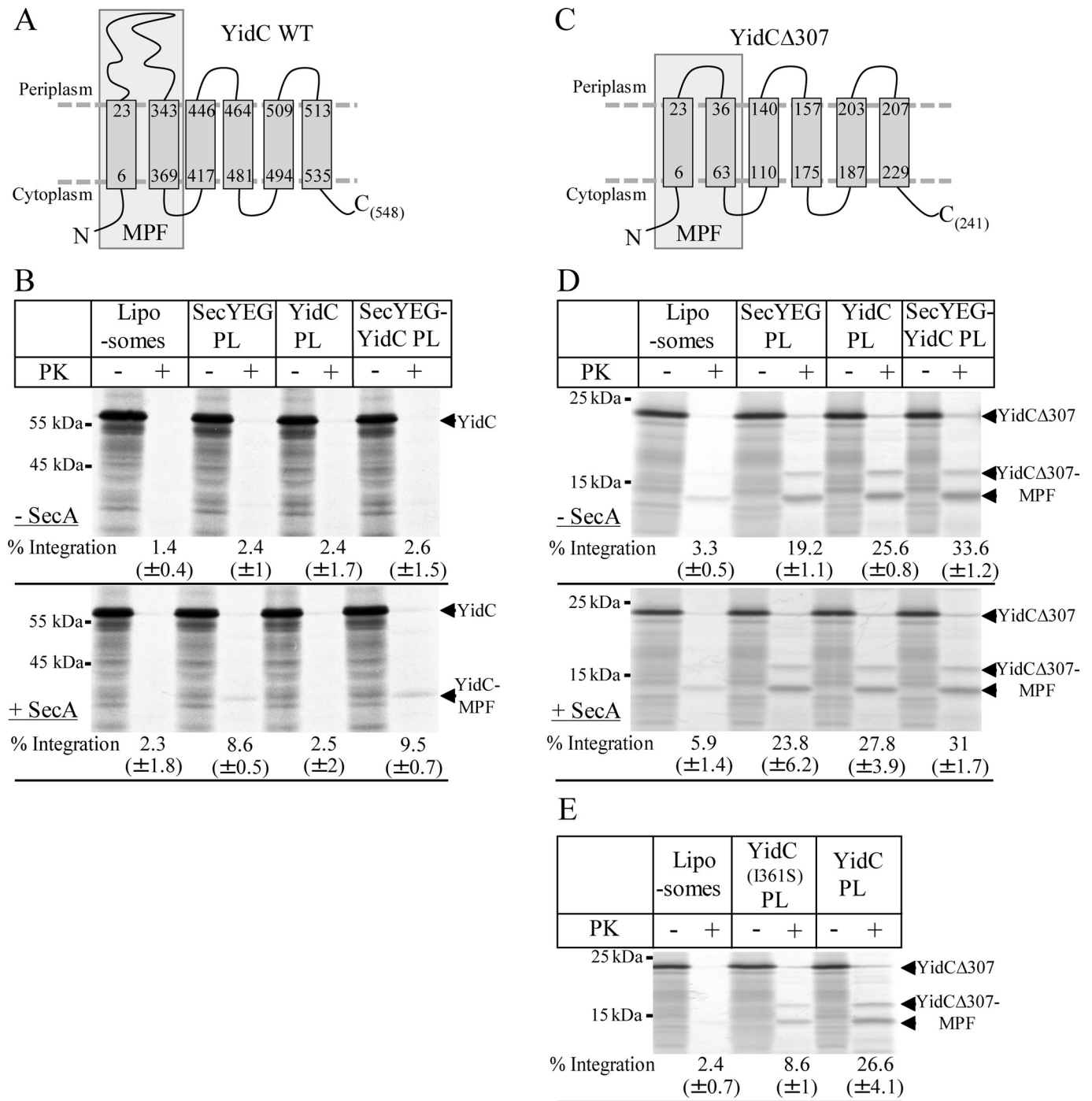
pholipids (Nishiyama *et al.*, 2006; Braig *et al.*, 2011). In SecYEG proteoliposomes, MtlA integration was more efficient and the level of integration was strongly increased when SRP/FtsY was added (Figure 4C). The latter finding is consistent with previous studies demonstrating that efficient targeting of MtlA to the SecYEG translocon is SRP dependent (Koch *et al.*, 1999). However, integration of MtlA into SecYEG proteoliposomes occurred also to some extent in the absence of SRP/FtsY, demonstrating that RNCs can be targeted to the SecYEG translocon independent of SRP *in vitro*. Different lengths of MtlA-RNCs have been shown to contact SecY and YidC sequentially, which has led to the hypothesis that YidC facilitates the release of transmembrane domains from SecYEG into the lipids (Beck *et al.*, 2001). This SecYEG-associated function of YidC does not seem to be essential for MtlA integration, as the MPF was also observed in the absence of YidC (Figure 4C; Braig *et al.*, 2011). Furthermore, coreconstituting SecYEG and YidC into proteoliposomes did not significantly increase MtlA integration (Figure 4C), although these proteoliposomes showed enhanced SRP-independent targeting. We also tested MtlA integration into proteoliposomes containing only YidC and found that MtlA was integrated into these proteoliposomes in the presence of SRP and FtsY (Figure 4C) but appeared to be less dependent on FtsY than on SRP, as it was also observed for TatC. MtlA was also not efficiently integrated into YidC(I361S) proteoliposomes (Figure 4D), suggesting that specific contacts between YidC and MtlA are required for integration.

Our data indicate that membrane proteins with multiple TMs like MtlA or TatC, which were previously believed to be exclusively inserted via the SecYEG translocon, can also be inserted by YidC. Of importance, targeting of substrates to either of the two translocases is mediated by SRP.

**YidC-dependent integration is restricted to membrane proteins with small periplasmic loops**

To further characterize the substrate specificity of the YidC integrase, we used YidC as a substrate. Previous studies showed that YidC is targeted by SRP to the SecYEG translocon (Koch *et al.*, 2002; Urbanus *et al.*, 2002) and that the translocation of its 320-amino acid long periplasmic loop requires SecA (Figure 5, A and B). This was

verified in our proteoliposome approach, since protease protection of YidC in SecYEG proteoliposomes was observed only when SRP/FtsY and SecA were present (Figure 5B). The MPF of YidC corresponds to the first two TMs and the connecting periplasmic



**FIGURE 5:** Membrane protein integration via YidC is restricted to membrane proteins with small periplasmic loops. (A) Predicted topology of YidC. The cleavage site for PK is indicated. The YidC-MPF corresponds to the first two TMs and the connecting periplasmic loop (Koch *et al.*, 2002). (B) YidC was synthesized in presence of SRP, FtsY, and liposomes/proteoliposomes as indicated. Protease protection was analyzed in the absence or presence of 600 nM SecA. Full-length YidC and YidC-MPFs are indicated. (C) Predicted topology of YidC $\Delta$ 307 (Deitermann *et al.*, 2005). (D) Protease protection of YidC $\Delta$ 307. The lower, 13-kDa MPF was used for calculating integration rates. (E) Protease protection of YidC $\Delta$ 307 in the presence of proteoliposomes containing wild-type YidC or the YidC(I361S) mutant.

loop as determined previously (Koch *et al.*, 2002; Serek *et al.*, 2004; Deitermann *et al.*, 2005). No protease protection of in vitro-synthesized YidC was observed in proteoliposomes reconstituted with YidC only (Figure 5B), even in the presence of SecA. YidC was inserted in SecYEG/YidC proteoliposomes at equivalent amounts as into SecYEG proteoliposomes (Figure 5B). This observation fur-

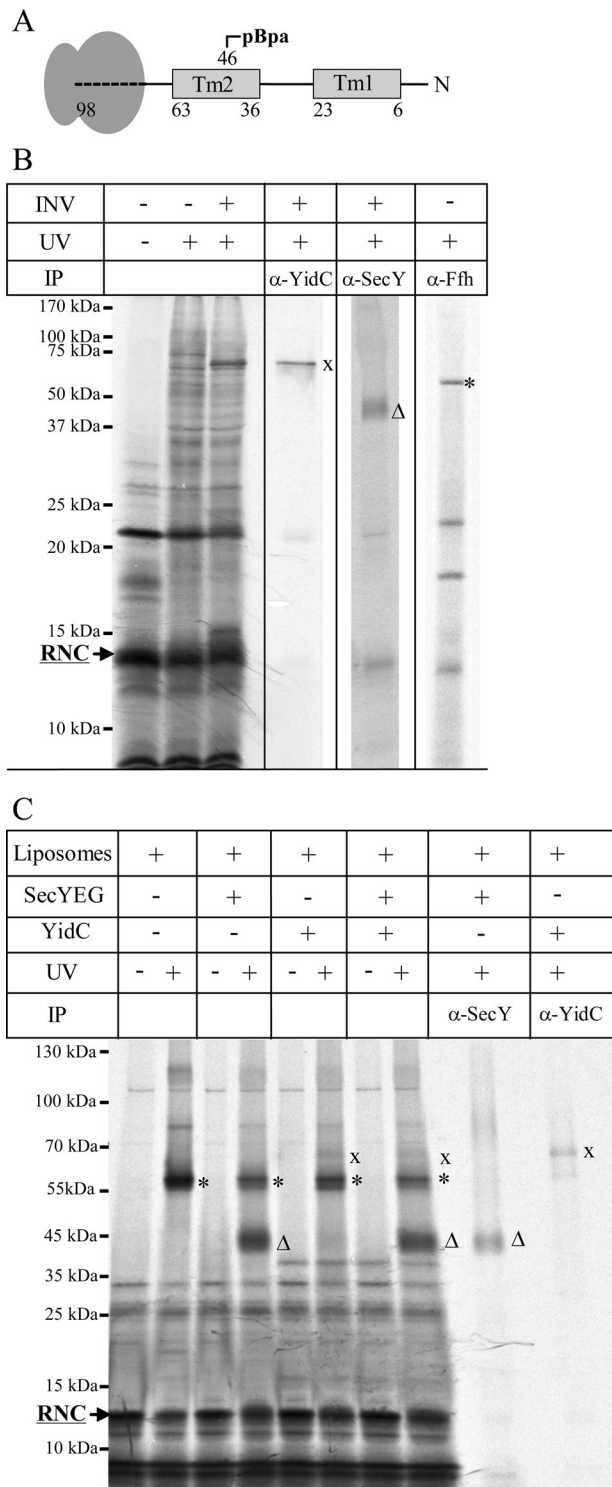
ther supports our conclusion that the SecYEG-associated function of YidC is not essential for membrane protein integration in vitro.

The observation that YidC proteoliposomes were able to integrate TatC and MtlA but not YidC suggests that the presence of large periplasmic loops impedes the integration via the YidC-only pathway. This was verified by using a YidC derivative in which the

coding region for residues 31–336 was deleted, resulting in a short, 13-amino acid long periplasmic loop between TM1 and TM2 (YidCΔ307; Figure 5C; Deitermann *et al.*, 2005). This derivative is integrated into INVs in a SecA-independent manner, yielding PK-protected MPFs of 18 and 13 kDa. The more prominent 13-kDa band represents the first two TMs plus the short periplasmic loop (Deitermann *et al.*, 2005), whereas the exact nature of the weaker 18-kDa band is unknown. Consistent with the data obtained with INVs, the integration of YidCΔ307 into SecYEG proteoliposomes did not require the addition of SecA (Figure 5D). YidCΔ307 was also efficiently integrated into YidC proteoliposomes and SecYEG/YidC proteoliposomes independent of SecA. We also observed that integration was reduced in YidC(I361S) proteoliposomes (Figure 5E). Similar to the result for MtlA, we observed a weak spontaneous insertion of YidCΔ307 into liposomes, probably reflecting penetration of TMs between the phospholipid head groups.

The integration of multispanning membrane proteins like YidCΔ307 by the YidC-only pathway was further analyzed by site-directed *in vitro* cross-linking using the UV-dependent cross-linker para-benzoyl-L-phenylalanine (pBpa). Previous INV studies showed that RNCs of membrane proteins cross-link to both SecY and YidC (Beck *et al.*, 2001; Urbanus *et al.*, 2001), suggesting that YidC is located in close proximity to SecY. This was also demonstrated *in vitro* for a 98-amino acid long RNC of YidCΔ307 (Figure 6A), which encompasses the first two TMs and harbors pBpa at position 46 within TM2. After UV exposure in the presence of INV, possible cross-links were identified by immunoprecipitations. We observed cross-links to Ffh at ~60 kDa (Figure 6B, asterisk), supporting SRP-dependent targeting of the YidCΔ307-RNCs to the membrane. We also observed cross-links to SecY at ~45 kDa and a strong cross-link to YidC at ~70 kDa (Figure 6B, triangle and cross). These data support a cooperative function of YidC in SecYEG-dependent membrane insertion. However, as YidCΔ307 can also be integrated by YidC-proteoliposomes (Figure 5D), we performed cross-linking experiments with proteoliposomes containing SecYEG, YidC, or both. After the addition of purified SRP and FtsY to allow targeting, we observed a strong cross-link of YidCΔ307-RNCs to Ffh at ~60 kDa on SDS-PAGE (Figure 6C, asterisk). In the presence of SecYEG proteoliposomes, the cross-link to Ffh became slightly weaker and an additional cross-link at ~45 kDa that was immunoprecipitated by α-SecY antibodies appeared (Figure 6C, triangle). In the presence of YidC proteoliposomes, the cross-link to Ffh was also slightly reduced, and a cross-linking product at ~70 kDa was detectable and recognized by α-YidC antibodies (Figure 6C, cross). However, the cross-link to YidC was significantly weaker than the cross-link to SecY. In proteoliposomes containing both YidC and SecYEG, the disappearance of the SRP cross-link was most pronounced and coincided with the appearance of both SecY and YidC cross-links.

We further analyzed whether insertion of membrane proteins via SecYEG or YidC was influenced by the membrane potential. The integration of MtlA, TatC, YidC, and YidCΔ307 into INV was not influenced by 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore that equalizes the proton concentration on both sides of the membrane (Supplemental Figure S2). We also did not observe a significant effect on the translocation of the SecA-dependent outer membrane protein OmpA (Supplemental Figure S2). These results are in line with studies showing a proton-motive force (pmf)-independent insertion of membrane proteins via SecYEG (Koch and Müller, 2000) or YidC (Yi *et al.*, 2004; van der Laan *et al.*, 2004b). In proteoliposomes, we found that YidC was integrated in a pmf-independent manner (Supplemental Figure S2). However, we were unable to determine a possible influence of the pmf on



**FIGURE 6:** Molecular contacts of YidCΔ307 RNCs in INV and proteoliposomes. (A) Cartoon showing the 98-amino acid long YidCΔ307-RNCs used in this study. YidCΔ307-98 RNCs comprise the first 98 amino acids of YidCΔ307 (Figure 5C) and contains pBpa at position 46 in TM 2. (B) YidCΔ307-98 RNCs were synthesized in the presence of INVs. Cross-linking products were identified by immunoprecipitations after separation on 15% SDS-PAGE. (C) YidCΔ307-RNCs were synthesized in the presence of SRP, purified via a sucrose cushion, and incubated with FtsY and liposomes/proteoliposomes as indicated. Samples were loaded on a 7–18% SDS gel. Cross-links to Ffh (asterisk), YidC (cross), and SecY (triangle) are indicated.



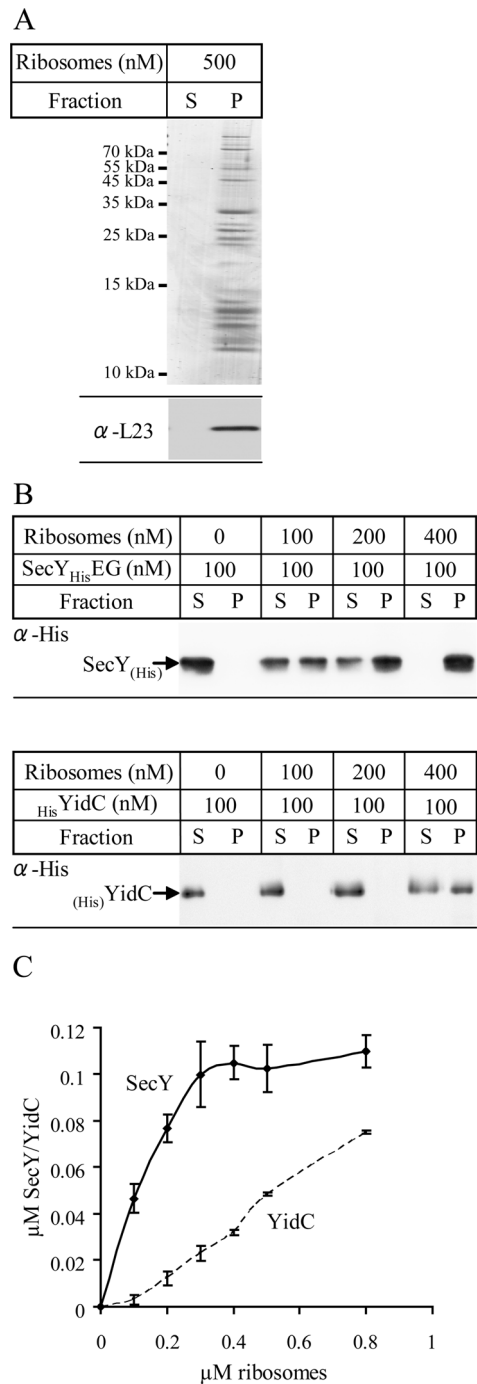
TatC and YidCΔ307 integration because both substrates probably aggregated in the buffer system used for energizing proteoliposomes and were not inserted.

In summary, our data demonstrate that membrane proteins like MtlA, TatC, and YidCΔ307 interact with and are integrated by YidC independent of SecYEG. However, YidC fails to insert membrane proteins with large periplasmic loops. The translocation of large periplasmic loops requires SecA. It is therefore likely that SecA-dependent membrane proteins can only be inserted by the SecYEG translocon.

### YidC binds to *E. coli* ribosomes

The observation that YidCΔ307-RNCs can be cross-linked to YidC indicates a cotranslational interaction, which is also supported by the stimulatory effect of SRP and the recent modeling of RNCs onto a YidC dimer (Kohler *et al.*, 2009). However, since YidC lacks the extended C-terminus that has been shown to be required for ribosome binding of Oxa1 or of the bacterial YidC2, we analyzed ribosome binding to YidC in a sedimentation assay. These assays were performed at pH 7.6 to prevent protonation of the N-terminal His tag. Sucrose gradient-purified *E. coli* 70S ribosomes were incubated with or without detergent-solubilized, purified, His-tagged YidC or SecY and centrifuged through a sucrose cushion. The amount of YidC or SecY in the supernatant and pellet fraction was then immunodetected using antibodies against their respective His tags. The use of antibodies against the His tag on these proteins allowed us to directly compare binding of ribosomes to YidC or SecY. Coomassie blue staining and immunodetection using antibodies against the ribosomal subunit L23 revealed that under these conditions ribosomal proteins were found in the pellet fraction (P) after centrifugation (Figure 7A). When SecY was incubated with increasing amounts of 70S ribosomes, we observed that most of SecY cosedimented with ribosomes even at a ribosome concentration of just 0.2 μM. At 0.4 μM ribosomes, SecY was almost completely bound to ribosomes and found in the pellet fraction. In contrast, SecY was exclusively recovered from the supernatant in the absence of ribosomes (Figure 7B). On the basis of these data we estimate that SecY binds to empty ribosomes with a  $K_D$  of ~0.15 μM (Figure 7C), which is in the range of the  $K_D$  values determined by Prinz *et al.* (2000). The same experimental setup was also used for measuring binding of YidC to *E. coli* ribosomes. Although binding of YidC to ribosomes was detectable, the affinity of YidC to empty ribosomes appeared to be significantly weaker than that of SecY (Figure 7B). In particular, no saturation was observed under the conditions tested, suggesting nonspecific binding. A rough estimation indicates that the affinity of YidC for ribosomes is ~5–10 times lower than the affinity of SecY for ribosomes (Figure 7C). It is also lower than the affinity of the Oxa1 C-terminus for mitochondrial ribosomes, which is in the range of 0.3–0.8 μM (Haque *et al.*, 2010a, 2010b).

The YidC-ribosome interaction was further verified *in vitro* by cross-linking experiments using sucrose gradient-purified INVs from *E. coli* cells expressing YidC with the cross-linker pBpa incorporated at position L540 at its C-terminus, YidC(L540pBpa). Cross-linking experiments with these membranes were performed without additional ribosomes, and we therefore determined contacts only to proteins that were purified together with INVs. On exposing YidC(L540pBpa) INVs to UV, several additional bands compared with the -UV control were detected by α-His antibodies, indicating that cross-linking had occurred (Figure 8A). Possible cross-links to ribosomal proteins were analyzed by Western blotting. The ribosomal protein L23 is located close to the ribosomal tunnel exit and was shown to be a major contact partner for SecY *in vivo* (Kuhn *et al.*, 2011). L23 was also proposed to be involved in YidC binding based



**FIGURE 7:** YidC binds to *E. coli* ribosomes. (A) Purified and salt-washed 70S ribosomes (500 nM) were incubated in binding buffer (pH 7.6). After 30 min of incubation on ice, ribosomes were centrifuged through a sucrose cushion. Pellet (P) and supernatant fractions (S) were separated on SDS gels and analyzed by Coomassie staining (top) and immunodetection using antibodies against L23 (bottom). (B) Increasing concentrations of salt-washed ribosomes were incubated with either 100 nM detergent-purified SecYEG (top) or detergent-purified YidC (bottom) in binding buffer (pH 7.6) and subjected to the same conditions as described in A. Pellet and supernatant fractions were immunodetected using antibodies against the N-terminal His tags of SecY or YidC. The percentage of binding was calculated using ImageJ software. (C) Quantification of at least three independent ribosome-binding experiments, using varying concentrations of SecYEG or YidC, respectively. The amount of ribosome-bound SecY/YidC was plotted against the total ribosome concentration.

on a recent cryo-EM study (Kohler *et al.*, 2009). However, antibodies against the ribosomal protein L23 did not detect any specific cross-linking product (Figure 8B). In contrast, INVs containing SecY with pBpa incorporated within the fifth cytosolic loop, SecY(R357pBpa), cross-linked efficiently to L23 under the same experimental conditions (Figure 8B). We also tested antibodies against L24 and L29, which, like L23, are located close to the ribosomal tunnel exit, and again we did not detect any cross-linking product (data not shown).

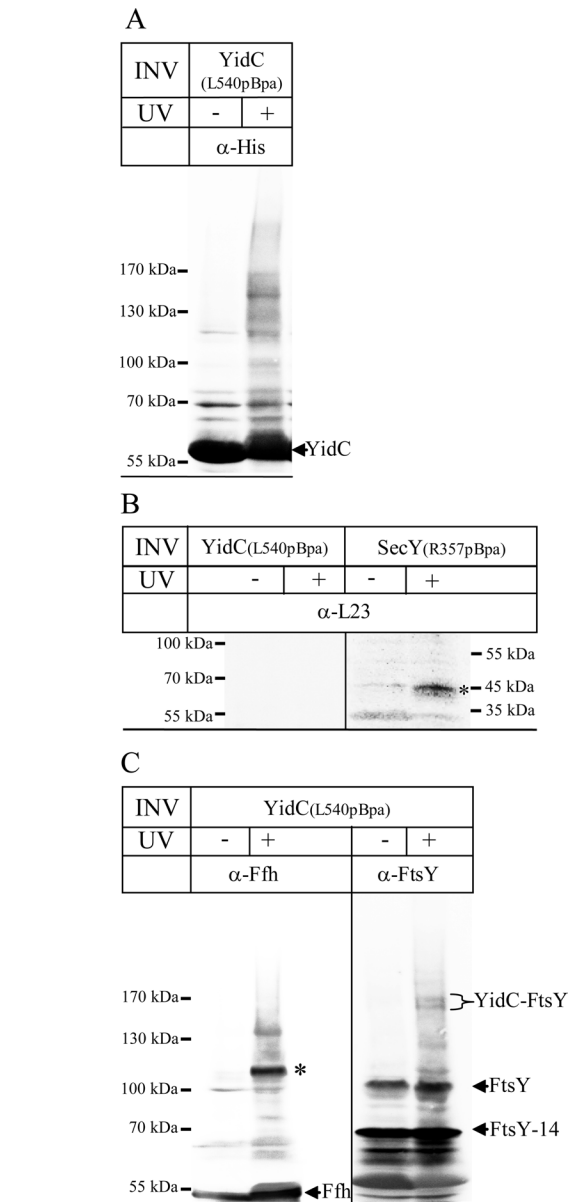
For increasing the sensitivity of our assay system, we analyzed gel lanes of UV-treated and untreated INVs by high-resolution mass spectrometry (MS). This approach identified several ribosomal subunits as cross-linking partners of YidC (Table 1). It is important to note that all ribosomal proteins listed were reliably identified in two independent cross-linking experiments and found at the expected size of the respective YidC cross-linking product in SDS-PAGE while being absent in the control sample (-UV). Consistent with our Western blotting data, cross-links to L23, L24, and L29 were not detected by MS (Table 1). We did, however, detect cross-links to L22, which is also located close to the ribosomal tunnel exit. The ribosomal subunits detected were identified with sequence coverage in the range of 16–58% and localized to both the large and small ribosomal subunits without distinct clustering. It is important to emphasize that in our approach we only probed for YidC contacts to nontranslating ribosomes. The presence of a signal-anchor sequence within YidC could stabilize the YidC-ribosome contact and allow for a more distinct cross-linking pattern involving the tunnel exit subunits. Additional ribosomal proteins were identified in only one of the two independent experiments (ribosomal subunits L3, L4, L7, L10, L11, L15, L27, L28, L30, L31, S5, S6, S7, and S10).

Combining cross-linking with MS allowed identification of cross-links between Ffh and YidC at 111 kDa in both experiments (Table 1). A cross-linked band at 110 kDa was also strongly recognized by  $\alpha$ -Ffh antibodies (Figure 8C), confirming the close proximity between Ffh and the C-terminus of YidC. We also detected cross-links between FtsY and YidC at ~160 kDa (Table 1), but a corresponding band was also detected in one experiment in the -UV control. Whether this is due to the aberrant migration behavior or to some contamination is unknown. Antibodies directed against FtsY recognized two strong bands at 70 and 95 kDa in both the UV-treated and untreated samples (Figure 8C). The 95-kDa band corresponds to full-size FtsY, whereas the 70-kDa band corresponds to an N-terminally truncated FtsY derivative (FtsY-14; Weiche *et al.*, 2008). In addition, two weak bands at ~160 kDa were recognized by  $\alpha$ -FtsY antibodies, which were only present in the UV-treated sample (Figure 8C). These bands most likely reflect cross-links of YidC to FtsY and FtsY-14, respectively. Additional UV-dependent bands at 110 and 130 kDa are probably nonspecifically recognized by the polyclonal  $\alpha$ -FtsY antibody (Figure 8C). By MS, we did not detect contacts between SecA and YidC. This could explain why YidC fails to integrate SecA-dependent membrane proteins (Figure 4).

In conclusion, the ribosome-binding assays and the cross-linking approach demonstrate that ribosomes can contact the C-terminus of *E. coli* YidC but that this interaction appears to be much weaker than the SecY-ribosome interaction. YidC also directly interacts with SRP and to a lesser extent with FtsY. Together with the proteoliposome results, this indicates that SRP can deliver multispansing membrane proteins not only to the SecYEG translocon, but also to the YidC integrase.

## DISCUSSION

Our data revealed four important functional aspects of YidC: 1) The SecYEG-associated function of YidC is not essential for the integra-



**FIGURE 8:** In vitro cross-links using INVs of YidC pBpa mutants show contacts to SRP and FtsY. (A) One mg of INVs from *E. coli* cells overexpressing YidC(L540pBpa) was resuspended in 250  $\mu$ l of INV buffer and cross-linked by UV irradiation. The samples were separated on a 5–15% SDS gel, and immunodetection was performed with  $\alpha$ -His antibodies. (B) Cross-linking with INVs purified from *E. coli* cells expressing YidC(L540pBpa) or the pBpa-containing SecY derivative SecY(R357pBpa), which contains pBpa within the fifth cytosolic loop of SecY. After separation on SDS-PAGE, cross-links to L23 (asterisk) were identified by immunodetection. (C) The material shown in A was also probed with  $\alpha$ -Ffh and  $\alpha$ -FtsY antibodies. The YidC-Ffh cross-link at 110 kDa is indicated (asterisk), as well as the Ffh that was present in the INVs. The YidC-FtsY cross-link appears in two distinct bands at ~160 kDa, which probably reflect the two FtsY isoforms present in *E. coli* INV. FtsY corresponds to full-size FtsY, whereas FtsY-14 corresponds to an N-terminally-truncated FtsY derivative.

tion of the membrane proteins. 2) YidC can function independent of SecYEG as an integration site for multispansing membrane proteins. 3) Only SecA-dependent membrane proteins strictly depend on SecYEG for integration. 4) SRP delivers membrane proteins to either SecYEG or YidC.

|                    | Protein <sup>a</sup> | Gel molecular weight (kDa) <sup>b</sup> | Molecular weight (kDa) <sup>c</sup> | Score <sup>d</sup> | Coverage <sup>e</sup> | Peptides <sup>f</sup> |
|--------------------|----------------------|---|-------------------------------------|--------------------|-----------------------|-----------------------|
| Ribosomal proteins | L5                   | 76.1                                    | 20.3                                | 120.8              | 58.1                  | 11                    |
|                    | L6                   | 76.1                                    | 18.9                                | 53.8               | 36.2                  | 6                     |
|                    | L13                  | 76.1                                    | 16                                  | 54.1               | 58.5                  | 8                     |
|                    | L14                  | 69.2                                    | 13.5                                | 78.9               | 28.5                  | 8                     |
|                    | L22                  | 69.2                                    | 12.2                                | 19.7               | 16.4                  | 3                     |
|                    | S11                  | 72.6                                    | 13.8                                | 23.8               | 27.9                  | 3                     |
|                    | S12                  | 75                                      | 13.7                                | 16.6               | 16.9                  | 2                     |
|                    | S13                  | 72.6                                    | 13.1                                | 28.1               | 33.1                  | 3                     |
|                    | S14                  | 69.2                                    | 11.6                                | 14.8               | 28.7                  | 2                     |
| Targeting factors  | Ffh                  | 111                                     | 49.8                                | 144.3              | 31.6                  | 13                    |
|                    | FtsY <sup>g</sup>    | 161                                     | 54.5                                | 14,84              | 3.82                  | 2                     |

Non-UV irradiated (not cross-linked, -UV) and UV-irradiated (cross-linked, +UV) samples of YidC(L540pBpa) INV were separated on a 5–15% SDS gel, and the proteins were visualized by Coomassie staining. The -UV and +UV lanes were cut into equal slices, followed by in-gel trypsin digestion and mass spectrometric analyses. Only ribosomal proteins that were specifically detected in two independent cross-linking experiments are listed.

<sup>a</sup>Protein identified.

<sup>b</sup>Molecular weight of gel slice determined by extrapolation.

<sup>c</sup>Calculated molecular mass.

<sup>d</sup>Protein score; in both replicates, a cut-off score of 5.5 corresponded to a protein false discovery rate of <0.01.

<sup>e</sup>Sequence coverage of total sequence by detected peptides.

<sup>f</sup>Number of peptides detected.

<sup>g</sup>FtsY/YidC-cross links were found specific in one experiment only.

**TABLE 1: Mass spectrometric analyses of YidC(L540pBpa) INV.**

YidC functions as an independent integrase for some membrane proteins but also assists the SecYEG translocon in membrane insertion. Proteins such as subunit K of NADH:ubiquinone oxidoreductase (Price *et al.*, 2010), subunit A of quinol oxidase (du Plessis *et al.*, 2006), and subunit A of ATP synthase (Yi *et al.*, 2004) require both SecYEG and YidC for insertion. Several functions have been assigned to YidC during SecYEG-dependent membrane insertion: it is suggested to chaperone membrane proteins (Nagamori *et al.*, 2004; Jong *et al.*, 2010), to facilitate the release of TMs from SecYEG (Beck *et al.*, 2001; Urbanus *et al.*, 2001; Houben *et al.*, 2004), to promote the formation of membrane protein complexes (Wagner *et al.*, 2008) and to control protein quality (van Bloois *et al.*, 2008). However, whether these SecYEG-associated functions of YidC are essential for membrane protein insertion was unknown. We now show that the integration of MtlA, TatC, and YidC via SecYEG does not require the assistance of YidC. The integration of these proteins into SecYEG proteoliposomes was almost as efficient as into proteoliposomes containing both SecYEG and YidC. The protease-protected fragments observed in the presence of SecYEG proteoliposomes were also identical to those observed in the presence of INV or SecYEG/YidC proteoliposomes. This indicates that MtlA and YidC, for which the MPFs are clearly defined (Werner *et al.*, 1992; Koch *et al.*, 2002; Deitermann *et al.*, 2005), are properly inserted by SecYEG in the absence of YidC. The observation that the SecYEG-associated function of YidC is not essential for membrane protein integration is consistent with studies on the single-spanning membrane protein FtsQ (van der Laan *et al.*, 2004b) and by studies showing that YidC depletion has only a minor effect on the integration of FtsQ or leader peptidase *in vivo* (Urbanus *et al.*, 2001; Fröderberg *et al.*, 2003).

So far, only single- or double-spanning membrane proteins with short periplasmic segments have been shown to be inserted via the YidC-only pathway, that is, independent of SecYEG (Xie and Dalbey,

2008). We now show that YidC can also insert multispanning membrane proteins like MtlA or TatC, which had been considered to be exclusively dependent on SecYEG. This is consistent with previous studies on TatC insertion in *E. coli* (Yi *et al.*, 2003) and chloroplasts (Martin *et al.*, 2009), where no clear dependence on either SecYEG or YidC for membrane insertion was demonstrated. Although YidC has been shown to improve membrane integration of TatC (Yi *et al.*, 2003), there was no effect of YidC depletion on the transport of Tat-dependent substrates (Hatzixanthis *et al.*, 2003). This suggests that TatC is inserted into the inner membrane either via SecYEG or YidC, as is also demonstrated by our PK protection and carbonate treatment assays. However, we cannot conclude which translocase takes precedence over the other while inserting TatC. Previous data from our lab on membrane protein insertion into SecYEG-depleted INVs show that although protein insertion is drastically reduced, there is some amount of insertion (Koch *et al.*, 1999; Koch and Müller, 2000; Deitermann *et al.*, 2005). This was previously attributed to membrane insertion via residual amounts of SecYEG, but considering our present data, it is likely that insertion occurred via YidC. This would also explain why SecA-dependent substrates appeared to be more sensitive toward SecYEG depletion than SecA-independent substrates (Koch *et al.*, 2002; Beha *et al.*, 2003), as YidC cannot transport SecA-dependent proteins. It is, however, important to mention that YidC is depleted during SecYEG depletion, since YidC has been shown to require SecYEG for its insertion (Koch *et al.*, 2002). Thus the YidC content of SecYEG-depleted INVs is extremely difficult to control.

In conclusion, these data suggest that the YidC-only pathway could be engaged by most bacterial membrane proteins, with the exception of membrane proteins containing large periplasmic loops. Because these proteins require SecA for the translocation of the periplasmic loops across the membrane (Deitermann *et al.*, 2005), it

is likely that SecA fails to directly interact with YidC, which is also consistent with our MS data.

Targeting of MtlA or TatC to YidC is strongly enhanced by SRP/FtsY. SRP-dependent targeting to YidC is supported by our cross-linking data, which show that SRP and to a lesser extent FtsY are in close proximity to the C-terminus of YidC. SRP release from the nascent chain by FtsY is a prerequisite for protein insertion into the SecY channel (Koch *et al.*, 1999), but insertion via YidC appears to proceed to some extent also in the absence of FtsY. It is possible that its direct interaction with YidC dissociates SRP from some nascent chains or that SRP release is induced by the RNC–YidC interaction. Finally, a possible posttargeting function of SRP has been proposed for SecY-dependent insertion (Bibi, 2012), which could also account for an SRP-dependent but FtsY-independent insertion via YidC. So far, direct interactions between SRP/FtsY and YidC homologues have only been observed for the chloroplast Alb3, which interacts with both FtsY (Moore *et al.*, 2003) and the chloroplast-specific SRP subunit cpSRP43 (Falk *et al.*, 2010; Lewis *et al.*, 2010; Richter *et al.*, 2010). Our data now show that SRP and FtsY are able to directly interact with YidC in bacteria. SRP-dependent targeting to YidC was previously also shown for MscL (Facey *et al.*, 2007) and F<sub>0</sub>C (van Bloois *et al.*, 2004). However, SRP targets F<sub>0</sub>C to the SecYEG translocon when YidC is absent (van Bloois *et al.*, 2004) and leader peptidase to SecYEG, as well as to YidC proteoliposomes (Houben *et al.*, 2002), although insertion can only occur via SecYEG. This indicates that SRP recognizes its substrates independent of downstream integration sites. Dual targeting by SRP occurs not only in bacteria but also in yeast, where SRP was shown to deliver substrates to both the Sec61 complex and the alternative Ssh1p translocon (Spiller and Stirling, 2011).

SRP-dependent targeting to SecYEG occurs cotranslationally and involves direct binding of RNCs to SecY. Whether ribosomes/RNCs can also bind to YidC has been debated, particularly because YidC lacks the extended C-terminus that was identified to be the ribosome-binding site in Oxa1 (Szyrach *et al.*, 2003). We now show that YidC binds to nontranslating ribosomes although with a lower affinity than SecY. Ribosome binding of YidC does not show saturation, indicating nonspecific binding, which also fits with the observation that YidC does not cross-link to a single ribosomal subunit. The C-terminus of YidC can be cross-linked to ribosomal proteins, but except for L22, we did not detect proteins that are located close to the tunnel exit. This is in contrast to a recent cryo-EM reconstruction study, which implicated proteins of the tunnel exit in YidC binding (Kohler *et al.*, 2009), very similar to the ribosome–SecY contact (Frauenfeld *et al.*, 2011). Because our *in vitro* cross-linking was performed with nontranslating ribosomes, whereas the cryo-EM study was performed with translating ribosomes, it is possible that the presence of a transmembrane domain influences the ribosome–YidC contact. It is likely that SRP promotes ribosome binding to YidC in *E. coli*, whereas mitochondria might have adapted to the lack of the SRP pathway by extending the C-terminus and providing a SRP-independent ribosome-binding platform for cotranslational insertion of membrane proteins. This would also explain why in *Streptococcus mutans*, the SRP pathway is not essential, as this organism contains two YidC homologues, one containing a C-terminal ribosome binding site (as in Oxa1) that allows cotranslational integration in the absence of SRP (Hasona *et al.*, 2005).

YidC cannot insert IMPs with large periplasmic loops, and therefore the SRP pathway needs to selectively target these proteins to SecYEG. Because SecA and FtsY transiently associate with SecY, it is possible that the presence of two receptors at the SecYEG translo-

con versus only one at YidC (FtsY) provides the necessary specificity. However, this would probably require that FtsY and SecA bind non-exclusively to the SecYEG translocon, which appears unlikely (Kuhn *et al.*, 2011). Alternatively, SecA-dependent membrane proteins that were targeted to YidC could use a mechanism similar to that proposed for CyoA insertion, which has been shown to require YidC, SecYEG, SecA, and the pmf for insertion (du Plessis *et al.*, 2006; Celebi *et al.*, 2006). It is possible that when YidC encounters SecA-dependent membrane proteins, it associates with the SecYEG translocon for allowing the passage of periplasmic loops. This needs to be further analyzed. It is also unknown why a small number of membrane proteins can only be inserted via YidC. This could be related to the hydrophobicity of the substrate. F<sub>0</sub>C has a mean hydrophobicity of 1.27 (calculated according to Kyte and Doolittle, 1982) and is exclusively YidC dependent, whereas TatC (0.75) and MtlA (0.33) are relatively less hydrophobic and can be inserted either via SecYEG or YidC. Dual targeting could also be dependent on charge distribution, given that it was recently shown for both NuoK (Price and Driessen, 2010) and M13 coat protein derivatives (Stiegler *et al.*, 2011) that insertion via YidC is influenced by the presence of negatively charged residues.

It is important to emphasize that our conclusions are mainly based on proteoliposome studies, and thus it is difficult to determine to what extent dual targeting occurs *in vivo*. However, recent proteome analyses support the theory of dual targeting *in vivo*. In SecE-depleted *E. coli* membranes that are also depleted in SecY, it was found that almost all outer membrane proteins were reduced, whereas a large number of inner membrane proteins appeared to be unaffected (Baars *et al.*, 2008). These inner membrane proteins lacked extended periplasmic loops, supporting our *in vitro* observation that multispinning membrane proteins with closely spaced TMs that are SecA independent can use YidC as an integration site. A proteome analysis of YidC-depleted membranes demonstrated that primarily the steady-state levels of membrane proteins with small soluble loops <100 amino acids are reduced (Wickström *et al.*, 2011), which suggests that closely spaced membrane proteins are preferentially targeted to YidC. It is possible that access of these membrane proteins to YidC is stochastically favored, because *E. coli* contains ~2500 YidC molecules (Drew *et al.*, 2003) but only 300–500 SecYEG complexes that are also required for the transport of most secretory proteins. Because translation is a rather slow process (~10 amino acids per second), most SecYEG complexes would probably be occupied by translating ribosomes if SecYEG constituted the only integration site for membrane proteins. Using YidC as an additional integration site would ensure that sufficient SecYEG complexes are accessible for the translocation of secretory proteins.

## MATERIALS AND METHODS

### Strains and plasmids

*E. coli* DH5 $\alpha$  (Hanahan, 1983), BL21, Top10 (Invitrogen, Karlsruhe, Germany), and MC4100 (Koch *et al.*, 1999) strains were used for amplifying plasmids and for preparation of INVs where indicated. BL21, BL21 (DE3; Novagen, Bad Soden, Germany), and TY0 (Taura *et al.*, 1997) were used for protein purification. pTrc99a-SecY<sub>His</sub>EG (Kuhn *et al.*, 2011) was used to prepare SecY<sub>His</sub>EG-overexpressing inverted membrane vesicles for *in vitro* cross-linking experiments and for purifying SecY<sub>His</sub>EG used in ribosome-binding assays.

The following plasmids were used for *in vitro* protein synthesis: p717MtlA (MtlA; Beck *et al.*, 2000), pDMB (OmpA; Behrmann *et al.*, 1998), pKSM-YidC and pKSM717-YidC $\Delta$ 307 (YidC, YidC $\Delta$ 307; Deitermann *et al.*, 2005), and pJM8CS7-SecY (Koch *et al.*, 1999).

tatC was amplified from the genome of *E. coli* MC4100 using the following primers: forward, 5' ACGCAAAGCATATGTCTGTAGAAGACTCAACCGCTTATCACG 3'; reverse 5' CCGCTCGAGTTATTCTCAGTTTTTTCGCTTTCTGCTTCAG 3'. The PCR product was digested with *NdeI/XhoI* and integrated into the *NdeI/XhoI*-digested pET19b, resulting in pET19b-TatC encoding an N-terminally His-tagged TatC. The N-terminal His tag of TatC was removed using the following primers: forward, 5' ATGTCTGTAGAAGACTCAACCGCT 3'; reverse, 5' GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTT 3'. TatC $\Delta$ N ( $\Delta$ 2-111) was constructed using 5' phosphorylated primers: forward, 5' GTTTCCAGCTCTCTGCTGTTTTATATCG 3'; reverse, 5' CATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTT 3'. TatC $\Delta$ C ( $\Delta$ 190-257) was constructed using 5' phosphorylated primers: forward, 5' TAACTCGAGGATCCGGCTGCTAACA 3'; reverse, 5' TAAGTCTTCTGGCGAGGTAATCCCAT 3'.

YidC was cloned from pROEX-HTB-YidC (Samuelson *et al.*, 2000) into pET19b using *NdeI/XhoI* restriction sites. His<sub>6</sub>YidC was then cloned into pTrc99a using *EcoRI/NcoI*, resulting in pTrc99a<sub>His</sub>YidC.

Mutational PCR was carried out using the Finnzymes Phusion PCR Kit (NE Biolabs, Frankfurt, Germany) and 5' phosphorylated primers: pTrc99a-YidC(L540pBpa) (forward, 5' GAAAACGTGGCTAGCATAGCCG 3'; reverse, 5' CAGACCACGGTAAATCAGCTGCTG 3'); pTrc99a-YidC(I361S) (forward, 5' ATTATCATCAGCACCTTTATCGTTCTGTG 3'; reverse, 5' GGAGAAGCCCCAGTTACCCACA 3'). Mutations in YidC $\Delta$ 307 to introduce the amber stop codon TAG were created using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Agilent, Santa Clara, CA) with the following primers: pKSM717YidC $\Delta$ 307(G46pBpa), forward, 5' CCATAGCTTTGTGTAGAACTGGGGCTTC 3'; reverse 5' GAAGCCCCAGTTCTACCAAAGCTATGG 3'.

#### In vitro protein synthesis, cytosolic translation factors, and salt-washed ribosomes

Proteins were synthesized in vitro using a purified transcription/translation system composed of cytosolic translation factors (CTFs) and high-salt-washed ribosomes or an S135 cell extract (Müller and Blobel, 1984; Koch *et al.*, 1999). For in vitro cross-linking, the CTF/S135 were prepared from *E. coli* Top10 pSup-pBpaRS-6TRN (Ryu and Schultz, 2006). RNCs were synthesized as described (Beck *et al.*, 2000) using the following oligonucleotide: YidC $\Delta$ 307 RNC-98, 5' CAGCGCCATCATTCTGGCT 3'. The [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine labeling mix was obtained from PerkinElmer (Wiesbaden, Germany), and pBpa was obtained from Bachem (Bubendorf, Switzerland).

INVs of *E. coli* cells were prepared as described previously (Müller and Blobel, 1984) with the following modifications: for cells expressing pBpa-containing proteins, the INV medium was supplemented with 1 mM pBpa (Chin *et al.*, 2002) and glucose was replaced by glycerol. Cells were cultivated at 30°C, induced at an OD<sub>600</sub> of 1 with 1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) for 5 h, and harvested.

#### Protein purification

The SecYEG complex was purified from p<sub>His</sub>EYG using a His-tagged version of SecE (Collinson *et al.*, 2001) in TY0 (MC4100) as described in Braig *et al.* (2011). For ribosome-binding assays, SecY<sub>His</sub>EG was purified following the same protocol. The proteins were visualized on an SDS gel after Coomassie staining, and the protein concentration was then adjusted to 0.4 mg/ml.

YidC and YidC(I361S) were purified from BL21 cells harboring pTrc99a-YidC or pTrc99a-YidC(I361S). Cells were grown at 180 rpm and 30°C up to an OD<sub>600</sub> of 1 before induction with 1 mM IPTG for 5 h. Purification was carried out as described for SecYEG by Braig

*et al.* (2011). The imidazole concentration used in the washing buffer was 40 mM. Proteins were visualized on Coomassie-stained SDS-PAGE, and the concentration was adjusted to 0.4 mg/ml.

SecA was purified of BL21 DE3 harboring pET19b SecA with an N-terminal 10xHis tag (Beha *et al.*, 2003). Cells were grown in 3 l of LB (lysogeny broth) medium to an OD<sub>600</sub> of 0.8 and were induced overnight with 0.5 mM IPTG. The cells were pelleted, resuspended, and broken using a French pressure cell. The lysate was centrifuged for 30 min at 30,000  $\times$  g to spin down unbroken cells. Purification was carried out as described for SecYEG by Braig *et al.* (2011). The protein was concentrated using a 50-kDa centrifugal filter (Amicon Ultra, Witten, Germany) and gel filtrated using an Äkta chromatography system and a Superdex 200 10/30 global column. The protein was dialyzed in CTF buffer and concentrated to 1.5 mg/ml.

pTrc99a-FtsY was purified as described previously (Braig *et al.*, 2009). pTrc99a-Ffh was purified as described (Braig *et al.*, 2011). Ffh was concentrated on a 10-kDa centrifugal filter (Amicon Ultra) and rebuffed in HT buffer plus 50% glycerol (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.6, 100 mM potassium acetate [KOAc], pH 7.5, 10 mM magnesium acetate [Mg(OAc)<sub>2</sub>], 1 mM dithiothreitol [DTT]) using a PD-10 column (GE Healthcare, Munich, Germany). The concentration of Ffh was 0.6 mg/ml. The protein was stored at -20°C. FtsY was rebuffed in HT buffer using a PD-10 Column (GE Healthcare). The concentration was adjusted to 1.4 mg/ml.

#### Preparation of proteoliposomes

*E. coli* phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Preparation of DAG and of liposomes containing 5% DAG was performed as described (Nishiyama *et al.*, 2006). Proteoliposomes were prepared by solubilizing 200  $\mu$ g of 5% DAG liposomes and 14–16  $\mu$ g of purified SecYEG or YidC in 150  $\mu$ l of 50 mM triethanolamine acetate (TeaOAc), pH 7.5, 1 mM DTT, and 1.5% octylglycoside. The samples dialyzed with 50 mM TeaOAc, pH 7.5, and 1 mM DTT. The proteoliposomes were pelleted and resuspended in 50 mM TeaOAc, pH 7.5, and 1 mM DTT. The proteoliposomes were stored at -80°C. Before each use, proteoliposomes were briefly sonicated. The generation of energized proteoliposomes followed established protocols (Serek *et al.*, 2004; van der Laan *et al.*, 2004b). In brief, proteoliposomes were resuspended after dialysis in 100 mM Na<sub>2</sub>SO<sub>4</sub> and sonicated. These proteoliposomes were added to the in vitro integration assay containing 100 mM KOAc. A transmembrane potential was established after the addition of 0.4  $\mu$ M valinomycin.

#### Preparation of 4.5S RNA

For in vitro synthesis of 4.5S RNA, pT7/T3 $\alpha$ 19, carrying the 4.5S RNA coding sequence (Wood *et al.*, 1992) was linearized using *Bam*HI. In vitro transcription was performed using the AmpliScribe T7-Flash Transcription kit (Epicentre Biotechnologies, Madison, WI). The 4.5S RNA was purified using the Norgen RNA Clean-Up and Concentration Kit (Norgen Biotec, Thorold, Canada). The final concentration of the RNA was adjusted to 0.7  $\mu$ g/ $\mu$ l and stored at -80°C.

#### In vitro INV pBpa cross-linking

For the cross-links with INVs, 1 mg of INVs were diluted to 4  $\mu$ g/ $\mu$ l in INV buffer (50 mM TeaOAc, pH 7.5, 250 mM sucrose, 1 mM DTT) and incubated for 20 min on ice. INVs were cross-linked on ice by UV irradiation for 20 min. INVs were trichloroacetic acid (TCA) precipitated and visualized by Western blotting or analyzed using mass spectrometry.

## Mass spectrometry

SDS–PAGE lanes were cut into horizontal 2-mm slices that were subjected individually to in-gel digestion by trypsin for establishing abundance profiles of identified peptides as described (Kuhn *et al.*, 2011). Peptides were analyzed by nano high-performance liquid chromatography (HPLC)–electrospray tandem mass spectrometry using the UltiMate 3000 HPLC system (Dionex LC Packings, Idstein, Germany) online coupled to an LTQ–Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany) as described previously (Kaller *et al.*, 2011). Proteins were identified by database searches against *E. coli* protein sequences deposited at the UniProt database (release 2011\_02) using the program OMSSA (version 2.1.9; Geer *et al.*, 2004), filtered, and quantified as described in Kuhn *et al.* (2011). Peptide scores were derived from P values calculated by the OMSSA program as  $-10 \log_{10}(P)$ , and protein scores represent the sum of peptide scores of all different peptides identified.

## In vitro transport assays

Assays in the coupled in vitro transcription/translation system have been described in detail (Koch *et al.*, 1999). The plasmid-encoded substrate was transcribed using T7-RNA polymerase and translated in the CTF/ribosome system. INV or proteoliposomes (lipids 0.4 mg/ml, 430 nM SecYEG, 530 nM YidC) and targeting factors (SRP, 150 nM; Ffh, 4.5S RNA, 15  $\mu$ g/ml; FtsY 750 nM; SecA 600 nM) were added from the beginning of the synthesis. The synthesis was carried out in a buffer with 40 mM TeaOAc, pH 7.5, 70 mM KOAc, and 10 mM Mg(OAc)<sub>2</sub>. When indicated, CCCP was added at a final concentration of 100  $\mu$ M. The synthesis/transport was carried out for 30 min at 37°C. After synthesis, the transport assay was split into two parts. One part was directly TCA precipitated, and one part was digested for 25 min at 25°C using 0.5 mg/ml proteinase K, following TCA precipitation and SDS–PAGE. For carbonate extraction, in vitro–synthesized samples were incubated with 0.2 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.3, for 30 min at 4°C and subsequently centrifuged for 30 min at 50,000 rpm in a Beckmann TLA 55 rotor. The supernatants were neutralized with glacial acetic acid and precipitated with one volume of 10% TCA, and the TCA pellet was resuspended in SDS loading buffer. Pellets were directly dissolved in loading buffer and, like supernatants, separated by SDS–PAGE.

## In vitro proteoliposome cross-linking experiments using RNCs with incorporated pBpa

The synthesis of RNCs has been described (Behrmann *et al.*, 1998; Beck *et al.*, 2000). The synthesis of stalled ribosome-nascent chains with incorporated pBpa requires a purified *E. coli* extract from a strain with the pSup-pBpaRS-6TRN plasmid. The ribosome-nascent chain of the protein with pBpa was synthesized using DNA of the protein with an amber stop codon (TAG), an antisense oligonucleotide that hybridizes to the mRNA of the protein, thus blocking translation, RNaseH to digest the DNA/RNA hybrid, and an antisense RNA for blocking *ssrA* (Hanes and Plückthun, 1997) and pBpa (40  $\mu$ M).

The synthesis of the RNCs was carried out for 30 min at 37°C and 300 rpm in a buffer with 40 mM TeaOAc, 70 mM KOAc, and 12 mM Mg(OAc)<sub>2</sub>. SRP was added to the synthesis for efficient binding. After synthesis, the RNCs were spun down on a sucrose cushion (580 mM sucrose, 40 mM HEPES, pH 7.5, 70 mM KOAc, 6 mM Mg(OAc)<sub>2</sub>, 1 mM DTT, 0.1 mM spermidine). RNCs were resuspended in a buffer with the ion strength of the synthesis mix, and proteoliposomes and FtsY were added. The transport was carried out for 30 min at 37°C. The transport assays were split, and one

sample was cross-linked on ice by UV irradiation for 20 min (wavelength, 365 nm; VL-6.L; Vilber Lourmat, Torcy, France); the other sample was kept in the dark for the same time. The samples were TCA precipitated or used for immunoprecipitations.

## Immunoprecipitations

Immunoprecipitations were carried out as described (Ahrem *et al.*, 1989). Samples from the cross-linking reactions were denatured for 15 min at 37°C using 1% SDS for YidC and 0.5% SDS for Ffh and SecY. After denaturation, the samples were incubated for 2–14 h with antibodies bound to 10 mg of protein A–Sepharose (GE Healthcare) on a wheel at 4°C. The Sepharose was washed three times using 1 ml of detergent buffer (25 mM Tris, pH 6.8, 5 mM EDTA, 150 mM NaCl, 1% Triton-X100) and two times with 1 ml of 25 mM Tris, pH 6.8. To release the protein from the Sepharose, it was incubated for 15 min at 37°C with 35  $\mu$ l of SDS loading buffer and then loaded on an SDS gel.

## Ribosome-binding assays

Ribosome-binding assays were performed as described (Prinz *et al.*, 2000). High-salt-washed ribosomes and purified SecY or YidC were incubated at 4°C for 30 min in HKM100 buffer (50 mM TeaOAc, pH 7.6, 5 mM Mg(OAc)<sub>2</sub>, 100 mM KOAc, 1 mM DTT, 0.1 mM spermidine, and 0.1% *n*-dodecyl- $\beta$ -D-maltoside, with a total reaction volume of 30  $\mu$ l). Subsequently, the samples were centrifuged for 10 min at 15,000  $\times$  *g* to remove aggregates, and the supernatant was loaded on a 100- $\mu$ l sucrose cushion (580 mM sucrose in HKM100 buffer) and ultracentrifuged for 20 min at 70,000 rpm in a Beckmann TLA-100.3 rotor. The pellet was directly resuspended in SDS loading buffer. The supernatant was diluted twofold with HKM100 buffer and precipitated with an equal volume of 20% TCA. After 2 h of precipitation on ice, the sample was centrifuged for 15 min at 15,000  $\times$  *g* and the pellet was resuspended in SDS loading dye. After SDS–PAGE, gels were Coomassie blue stained or blotted and decorated with antibodies against ribosomal subunit L23, YidC, or SecY. For quantification, the protein amount in the pellet and supernatant after ultracentrifugation as seen in the immunoblots was determined using ImageJ software (National Institutes of Health, Bethesda, MD).

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