



SRP, FtsY, DnaK and YidC Are Required for the Biogenesis of the *E. coli* Tail-Anchored Membrane Proteins DjIC and Flk

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Abstract

Tail-anchored membrane proteins (TAMPs) are relatively simple membrane proteins characterized by a single transmembrane domain (TMD) at their C-terminus. Consequently, the hydrophobic TMD, which acts as a subcellular targeting signal, emerges from the ribosome only after termination of translation precluding canonical co-translational targeting and membrane insertion. In contrast to the well-studied eukaryotic TAMPs, surprisingly little is known about the cellular components that facilitate the biogenesis of bacterial TAMPs. In this study, we identify DjIC and Flk as *bona fide* *Escherichia coli* TAMPs and show that their TMDs are necessary and sufficient for authentic membrane targeting of the fluorescent reporter mNeonGreen. Using strains conditional for the expression of known *E. coli* membrane targeting and insertion factors, we demonstrate that the signal recognition particle (SRP), its receptor FtsY, the chaperone DnaK and insertase YidC are each required for efficient membrane localization of both TAMPs. A close association between the TMD of DjIC and Flk with both the Ffh subunit of SRP and YidC was confirmed by site-directed *in vivo* photo-crosslinking. In addition, our data suggest that the hydrophobicity of the TMD correlates with the dependency on SRP for efficient targeting.

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Introduction

Efficient targeting and insertion of integral membrane proteins is critical for both pro- and eukaryotic cells because their hydrophobic transmembrane domains (TMDs) are prone to aggregation in the aqueous cytosol and must therefore be shielded prior to insertion into the lipid bilayer. In the conserved co-translational targeting pathway, the signal recognition particle (SRP), a ribonucleoprotein complex, interacts with the TMD of a nascent membrane protein as soon as it emerges from the ribosome exit tunnel [1]. This allows ribosomes to directly transfer TMDs into the Sec protein translocase, residing in the plasma membrane of prokaryotes and in the endoplasmic reticulum of eukaryotes, from which they partition laterally into the lipid bilayer.

Intriguingly, a small subset of membrane proteins depends on a single C-terminal TMD for anchoring in the membrane. Consequently, the TMD of these so-called tail-anchored membrane proteins (TAMPs) emerges from the ribosome only after termination of translation thereby precluding their use of co-translational targeting and insertion mechanisms. Because TAMPs play a crucial role in important physiological processes, such as vesicular transport, their biogenesis in eukaryotes has been studied in great detail and several complementary pathways have emerged [2,3]. In both yeast and mammalian cells, a few TAMPs are capable of unassisted membrane insertion; however, most TAMPs follow one of up to four chaperone-mediated pathways depending in part on the hydrophobicity of the TMD [2]. One pathway is mediated by the SRP recruiting TAMPs with a relatively

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hydrophobic TMD in an unusual post-translational fashion, another uses the Hsc70/Hsp40 pairing, and a third the recently discovered SND (SRP-independent targeting) pathway [4]. The final option is the dedicated GET (Guided Entry of TA proteins) pathway, which appears to be prevalent for the targeting and insertion of TAMPs into the membrane of the endoplasmic reticulum [2]. Despite the predicted existence of TAMPs in bacteria, surprisingly little is known about their biogenesis [3].

To start our analysis of the targeting and insertion of bacterial TAMPs, we considered DjIC and Flk [5,6] that differ in the hydrophobicity of their predicted TMDs as model TAMPs. Differential centrifugation and membrane extraction experiments confirmed that both model proteins are *bona fide* *Escherichia coli* TAMPs and require their predicted C-terminal TMD sequence for proper membrane insertion. Further analysis by fluorescence microscopy showed that the TMDs of DjIC and Flk are sufficient to target a soluble, fluorescent reporter protein to the membrane. Using strains conditional for the expression of known targeting and insertion factors, we found that SRP, FtsY, DnaK and YidC are crucial for successful localization of the reporter TAMP fusion constructs.

Results

Identification of potential *E. coli* TAMPs

An algorithm has been described to predict prokaryotic TAMPs based on the presence of a single predicted TMD not more than 30 amino acids from the C-terminus of the protein and the absence of a predicted N-terminal signal sequence [3]. Based on this algorithm, the genes for three candidate TAMPs (DjIC, Flk, MnmG) were cloned in a pET expression vector and checked for expression. Based on stable, non-toxic expression (not shown), DjIC and Flk were selected as model TAMPs for further studies. A second argument for this choice is the difference in hydrophobicity of the predicted TMDs of DjIC and Flk. In yeast and mammalian cells, the hydrophobicity of the tail-anchor is an important parameter for the preferred targeting pathway [7]. DjIC is a 56-kDa DnaJ-like protein that functions as a co-chaperone of HscC [5] and has a moderately hydrophobic TMD of 1.95 GRAVY (Grand Total Average of Hydropathy[†]) comparable to the eukaryotic TAMP Sec61 β (2.05 GRAVY). Flk is a 37-kDa regulator of flagellar gene expression [6] with a very hydrophobic tail-anchor of 2.83 GRAVY, similar to Synaptobrevin-2, a eukaryotic TAMP with a TMD of 2.82 GRAVY.

DjIC and Flk are integral inner-membrane proteins

To determine the subcellular localization, cells expressing FLAG-tagged DjIC or Flk were lysed and

different cell fractions were obtained using differential centrifugation. As seen in Fig. 1b, both DjIC and Flk were detected in the high-speed pellet fraction that contains crude membranes, co-fractionating with the integral inner-membrane control proteins leader peptidase (Lep) and SecG. As negative controls for the localization studies, we created mutant derivatives of both proteins that lack the predicted C-terminal TMD and are expected to remain in the cytoplasm (Fig. 1a).

As expected, Flk Δ TMD was almost exclusively detected in the high-speed supernatant that contains cytoplasmic and periplasmic proteins (Fig. 1b). The slightly lower expression level compared to the wild-type Flk might be due to instability of the mislocalized protein. In contrast, DjIC Δ TMD was mainly found in the high-speed pellet, suggesting that DjIC contains intrinsic sequence information other than the TMD to associate with the membrane. Alternatively, DjIC Δ TMD could form aggregates in the cytoplasm that co-fractionate with the membranes during centrifugation. To address this issue, the high-speed pellet fractions were further analyzed by sucrose density centrifugation (Fig. 1c). As expected, wild-type DjIC was primarily seen in the fractions that also contained the control inner-membrane protein YidC in between the fractions in which the soluble control protein RNA polymerase α (RpoA) and the outer-membrane protein OmpA were peaking. In contrast, DjIC Δ TMD was roughly equally distributed in all gradient fractions. Although we cannot exclude from these data that DjIC Δ TMD is in part associated with the inner membrane, the widespread distribution in density rather suggests that it accumulates in aggregates of variable sizes.

To investigate the nature of the association of the model TAMP DjIC with the inner membrane in more detail, membranes were extracted with different agents. Na₂CO₃, which is commonly used to remove peripheral membrane proteins [8], did not affect the distribution of DjIC, whereas the detergent *N*-dodecyl- β -D-maltoside (DDM) at 2% resulted in partial solubilization of DjIC but less than the control inner-membrane protein Lep (Fig. 2a). These data argue that DjIC is an integral membrane protein but part of the expressed DjIC ends up in DDM-insoluble aggregates. In contrast, DjIC Δ TMD was almost completely insoluble upon extraction with both Na₂CO₃ and DDM (Fig. 2a), suggesting that the aggregates formed are quite rigid. To examine the nature of the aggregates in more detail, the high-speed pellet fraction of DjIC Δ TMD was extracted with urea which is known to dissolve most protein aggregates at higher concentrations [9]. Indeed, using 6 M urea, almost all DjIC Δ TMDs were dissolved while leaving integral membrane proteins mostly unaffected as seen for the control protein SecG that was only partially extracted at 8 M urea (Fig. 2b).

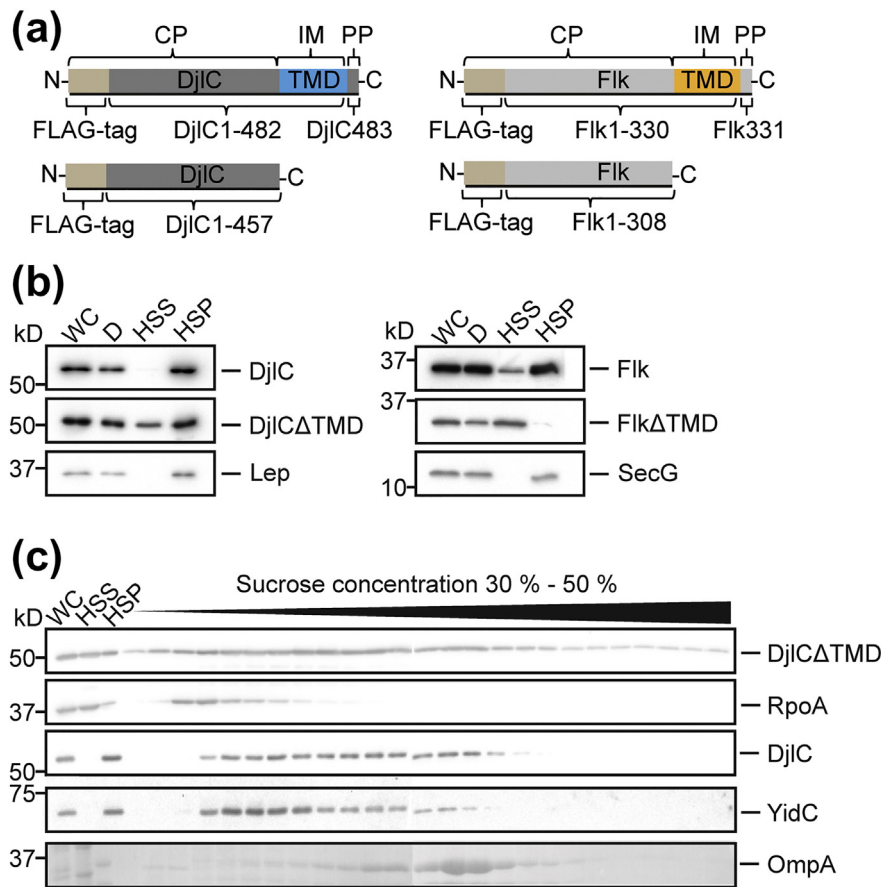


Fig. 1. DjIC and Flk co-fractionate with membranes. N-terminally FLAG-tagged DjIC, Flk and mutants lacking the TMD were expressed in *E. coli* TOP10F'. After expression, cells were lysed and subjected to differential centrifugation. (a) Schematic representation of the constructs and their predicted topology indicating the domains located in the cytoplasm (CP), inner membrane (IM) or periplasm (PP). (b) Whole cell (WC), debris (D), high-speed supernatant (HSS) and high-speed pellet (HSP) fractions were analyzed by Western blotting. (c) Crude membranes of cells expressing FLAG-tagged DjIC or DjICΔTMD were subjected to sucrose gradient centrifugation. Fractions were taken from the top of the gradient to the bottom and TCA-precipitated. Whole cell (WC), high-speed supernatant (HSS), high-speed pellet (HSP) and sucrose gradient fractions were analyzed by Western blotting. TAMP constructs were detected using FLAG antibodies. RpoA and Lep and SecG were detected by specific antibodies to serve as cytoplasmic and inner-membrane reference protein, respectively. The outer-membrane protein OmpA was detected by Ponceau staining.

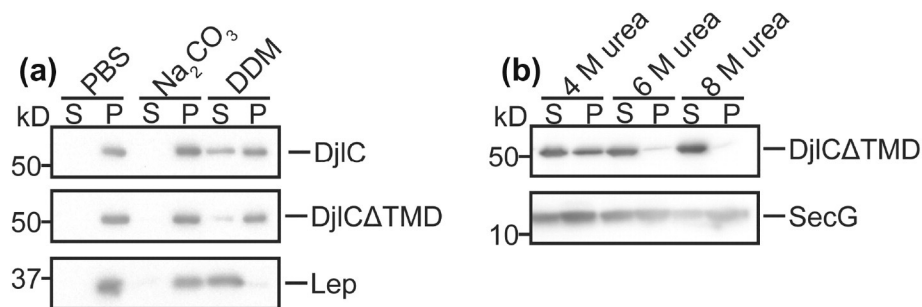


Fig. 2. DjIC that lacks its TMD is prone to aggregation. Crude membranes of *E. coli* TOP10F' expressing FLAG-tagged DjIC and DjICΔTMD were isolated by differential centrifugation. Crude membranes were treated with (a) PBS, 0.2 M Na₂CO₃ or 2% DDM or with (b) 4, 6, or 8 M of urea and subjected to high-speed centrifugation. Supernatant fractions (S) were TCA-precipitated and together with pellet fractions (P) analyzed by Western blotting using FLAG antibodies. Lep and SecG served as inner-membrane protein controls as described in Fig. 1.

The TMDs of DjIC and Flk are sufficient to target mNeonGreen to the membrane

The data described above show that biochemical fractionation of TAMPs is prone to artifacts. In particular, it appears difficult to reliably distinguish between membrane localized and aggregated material in fractionation experiments. In order to develop an independent, more direct method to determine the subcellular localization of TAMPs, we created fluorescent chimeras for analysis by fluorescence microscopy. The bright fluorescent protein mNeonGreen (NG) [10] was fused at its C-terminus to the TMD and predicted periplasmic tail of DjIC or Flk, using approximately 20 residues upstream of the TMD as linker sequence (Fig. 3a). In addition, NG was supplied with a His-tag at its N-terminus for detection and purification creating His-NG-DjIC-TMD and His-NG-Flk-TMD, respectively. Analyzing *E. coli* TOP10F' cells expressing either of these constructs showed that both fluorescent NG chimeras form a distinct halo-shaped signal at the cell envelope, indicating membrane localization (Fig. 3b). In contrast, unfused NG,

which was used as a negative control, showed a disperse signal throughout the cell, as expected for a protein that resides in the cytoplasm (Fig. 3b). Further analysis using subcellular fractionation showed that both NG-DjIC-TMD and NG-Flk-TMD co-localize with crude membranes, whereas NG is found in the soluble fraction (Fig. 3c).

Of note, the predicted C-terminal periplasmic "tail" of both DjIC and Flk only consists of a single amino acid. To confirm that the C-terminus of the NG fusion constructs is translocated across the inner membrane, we created new constructs suitable for proteinase accessibility assays. Both NG-DjIC-TMD and NG-Flk-TMD were provided with an N-terminal FLAG-tag and a C-terminal 5-amino-acid-long flexible linker (GGGGS) followed by a 13-amino-acid-long Opsin-tag. Upon expression in the wild-type *E. coli* strain MC4100, the cells were converted to spheroplasts and treated with proteinase K (ProtK). As shown in Fig. 3d, detection of the FLAG-tagged domains was not affected upon ProtK treatment unless the cells were lysed with Triton, indicating that the constructs are not intrinsically ProtK resistant. Similar results

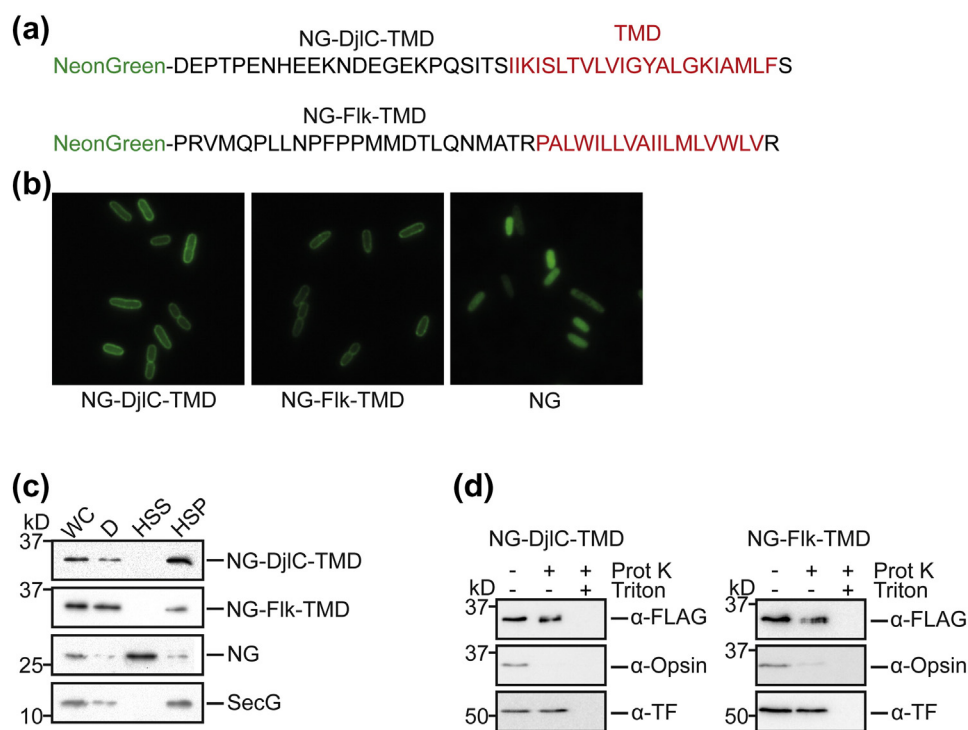


Fig. 3. Subcellular localization of fluorescent NG-TMD chimeras. His-tagged NG, NG-DjIC-TMD and NG-Flk-TMD were expressed in *E. coli* TOP10F'. (a) Amino acid sequences of the C-terminal regions of DjIC and Flk that were fused to the C-terminus of NG, respectively. (b) A portion of the cells was fixed with formaldehyde and analyzed by fluorescence microscopy. (c) The remaining cells were subjected to lysis and differential centrifugation and whole cell (WC), debris (D), high-speed supernatant (HSS) and high-speed pellet (HSP) fractions were analyzed by Western blotting. (d) Topology determination of N-terminally FLAG-tagged and C-terminally Opsin-tagged NG-TMD chimeras expressed in *E. coli* MC4100. The cells were converted to spheroplasts and incubated with ProtK, ProtK and Triton or buffer. Samples were analyzed by Western blotting. NG chimeras were detected using anti-His, anti-FLAG or anti-Opsin sera, whereas SecG served as reference inner-membrane protein as described in Fig. 1. TF served as loading control for the ProtK accessibility assay.

were obtained with the cytoplasmic control protein trigger factor (TF). In contrast, the ProtK treatment diminished detection of the Opsin-tag suggesting its exposure in the periplasm.

Combined, the results show that the TMDs of DjIC and Flk are capable of targeting a soluble, fluorescent protein to the membrane and inserting it in the expected C-out N-in topology, suggesting that the TMD is not only required but also sufficient for membrane localization of both DjIC and Flk in *E. coli*.

Ffh, FtsY, DnaK and YidC are required for successful membrane targeting of fluorescent NG-TMD model proteins

Since the TMD of TAMPs only emerges from the ribosomal exit tunnel after completion of translation, their targeting and insertion has to occur in a post-translational manner. In eukaryotes, most TAMPs follow one of four chaperone-mediated pathways depending on the hydrophobicity of the TMD [2,4]. Since little is known about the routing of TAMPs in *E. coli* and the factors involved, we started out by analyzing the requirement for known targeting and translocon components for proper localization of our model TAMPs. To this end, we expressed the

fluorescent chimeras NG-DjIC-TMD and NG-Flk-TMD in strains conditional for the expression of these factors and determined their localization by fluorescence microscopy. These factors included Ffh (protein subunit of SRP), FtsY (SRP receptor), DnaK (Hsp70 chaperone), SecE (part of the SecYEG translocon) and YidC (membrane insertase that can act both alone and in conjunction with the Sec-translocase) [11].

In eukaryotic cells, the SRP was shown to be involved in the targeting of TAMPs with a relatively hydrophobic TMD [7]. To investigate the role of the SRP in the targeting of TAMPs in *E. coli*, we used strain HDB51, in which the expression of *ffh* is controlled by an L-arabinose-inducible promoter [12]. Upon growth in the presence of arabinose (non-depleted conditions), both fluorescent NG chimeras show a halo-shaped signal at the cell envelope, indicating membrane localization comparable to a wild-type strain (Fig. 4a). In cells grown in the absence of arabinose (depleted of Ffh), we observed a minor effect on the localization of NG-DjIC-TMD. The halo-shaped signal is still evident, but disperse spotty signals appear that probably represent accumulation of mislocalized protein (Fig. 4a). However, the effect on the targeting of NG-Flk-TMD is more pronounced.

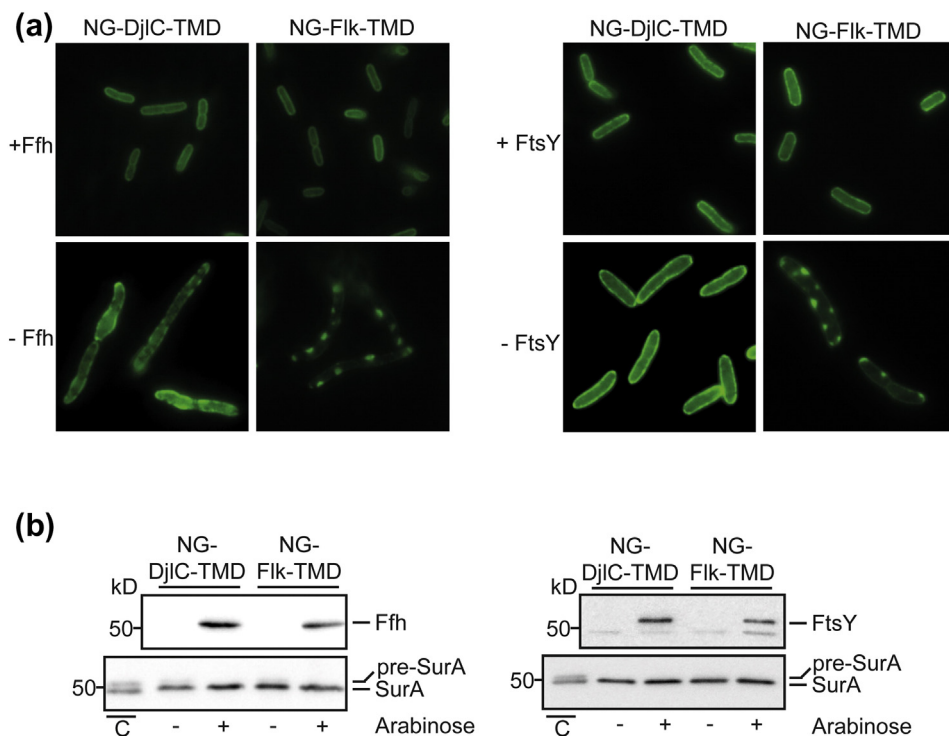


Fig. 4. Ffh and FtsY are required for efficient localization of NG-TMD chimeras. (a) NG-DjIC-TMD and NG-Flk-TMD were expressed under depleted and non-depleted conditions in strains conditional for the expression of Ffh and FtsY. Cells were fixed with formaldehyde and analyzed by fluorescence microscopy. (b) Whole cell samples were analyzed by Western blotting to verify the depletion of Ffh and FtsY with specific antibodies. The processing of the secreted protein SurA served as over-depletion control. The pre-SurA control sample was obtained from whole cells of the temperature-sensitive SecA strain MM52 grown under non-permissive conditions.

No halo-formation could be observed and large spots, probably aggregates accumulated in the cells, indicating a strong dependency on the SRP for proper targeting (Fig. 4a). As observed before [13], depletion of Ffh led to modest cell filamentation. Depletion of Ffh was further verified by Western blotting (Fig. 4b). To check if the depletion of Ffh caused secondary effects that might inactivate other targeting and insertion machineries, we monitored the processing of the Sec-dependent secreted protein SurA. No unprocessed form of SurA accumulated in the cells under the depletion conditions used, indicating that inactivation of the Sec-translocon had not occurred (Fig. 4b). To further investigate the involvement of the SRP pathway, both NG chimeras were expressed in the strain IY26 which is conditional for the expression of the SRP receptor FtsY. In non-depleted conditions, both NG chimeras display a halo at the cell envelope similar to wild-type cells (Fig. 4a). In cells depleted of FtsY, the localization of NG-DjlC-TMD remained unaffected. In contrast, however, the localization of NG-Flk-TMD was severely affected and large spots appeared at various positions in the cells similar to the effect of Ffh depletion (Fig. 4a). Western blot analysis confirmed the depletion of FtsY (Fig. 4b) and the processing of the secreted protein SurA was unaffected, indicating that over-depletion had not occurred (Fig. 4b). To gain more insight into the characteristics of the spotty signals, the fluorescence images were further analyzed with “Coli-Inspector,” a project running under the image processing program ImageJ within the “ObjectJ” plugin [14]. This software automatically detects the amount and positions of the

fluorescent spots within the cells. Plotting the positions of the spots within the cells against the cell lengths revealed that the spots of both NG chimeras distribute along the length of the cells (Fig. S2a–d). Examining the percentage of cells that contain 0, 1, 2 or ≥ 3 spots, it appeared that the accumulation of spots under Ffh- and FtsY-depleted conditions is higher for NG-Flk-TMD compared to NG-DjlC-TMD (Fig. S2a–d) indicating a stronger dependency on an intact SRP pathway.

A subset of eukaryotic TAMPs with mildly hydrophobic TMDs requires Hsp70 for biogenesis [15]. Since the *E. coli* Hsp70 (DnaK) is not essential at lower temperatures, we could verify its role by simply expressing the NG chimeras in a DnaJ/K knockout strain and its isogenic wild-type (MC4100) as control. As expected, both chimeras successfully localized at the cell envelope in the wild-type strain (Fig. 5a). In the absence of DnaJ/K, both chimeras showed spotty signals within the cells, indicating mislocalization of the protein, although the effect was clearly strongest for NG-DjlC-TMD (Fig. 5a). Interestingly, the spots were mostly located at the cell poles (Fig. S3a and b) different from the disperse pattern observed upon depletion of Ffh and FtsY (Fig. S2a–d). Since DnaK is a generic chaperone, we reasoned that its absence might lead to aggregation of the NG part of the chimeric TAMPs. To exclude this possibility, we investigated the localization of wild-type NG and NG fused to the N-terminus of full-length DjlC (NG-DjlC) and full-length Flk (NG-Flk) in the absence and presence of DnaJ/K. Wild-type NG displayed a diffuse fluorescent signal throughout both wild-type and

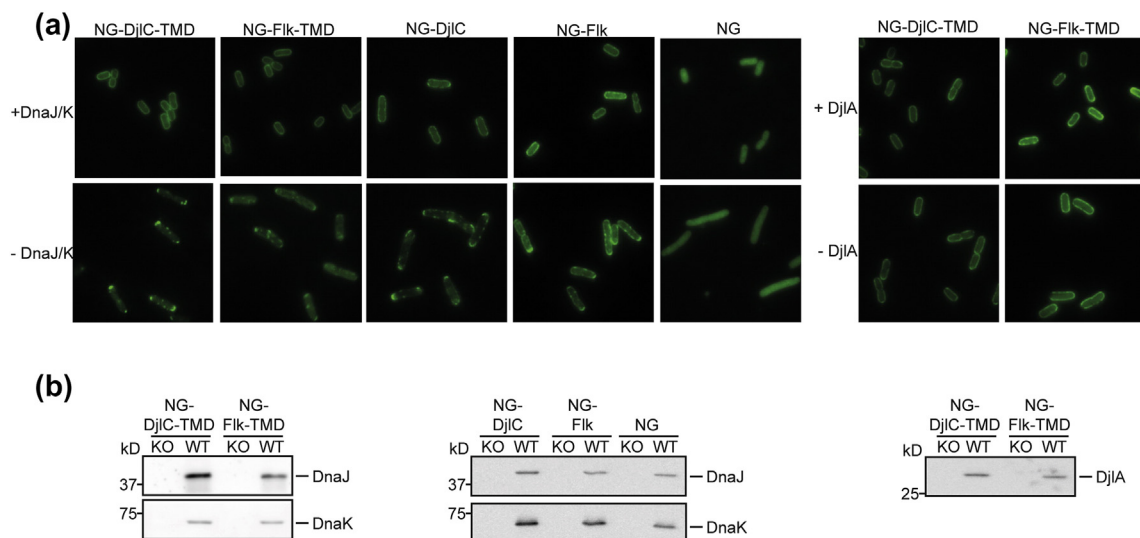


Fig. 5. DnaJ/K is required for efficient localization of NG-TMD chimeras. (a) NG-DjlC-TMD, NG-DjlC, NG-Flk-TMD, NG-Flk and NG were expressed in DnaJ/K and DjlA knockout strains and their isogenic wild-type strain MC4100. Cells were fixed with formaldehyde and analyzed by fluorescence microscopy. (b) The absence of DnaJ/K and DjlA in the knockout strains (KO) compared to the wild-type strain (WT) was confirmed by Western blotting of whole cell samples using specific antibodies.

DnaJ/K knockout cells (Fig. 5a), indicating that folding of NG does not require DnaJ/K. In contrast, both NG-DjlC and NG-Flk folded and localized properly in the wild-type strain but accumulated in spots when DnaJ/K was absent (Fig. 5a), indicating that DnaJ/K is required for the biogenesis of full-length DjlC and Flk. The absence of DnaJ/K in the knockout strain was verified by Western blotting (Fig. 5b). Since DnaJ/K appears to be required for TAMP biogenesis, we also investigated whether DjlA, the potential membrane receptor of DnaK [16], is involved in this process. We expressed both NG-DjlC-TMD and NG-Flk-TMD in a DjlA knockout strain and its isogenic wild-type (MC4100) but did not observe any difference in the membrane localization of the NG chimeras (Fig. 5a), indicating that DjlA is not required for biogenesis of the chimeras.

YidC plays an important role in the insertion and assembly of most inner-membrane proteins [11]. In particular, small membrane proteins that do not require the Sec-translocon are highly dependent on YidC for successful insertion *via* the so-called “YidC-only pathway.” To investigate the role of YidC in the insertion of the NG chimeras, we used the strain MK6s, in which the expression of the endogenous *yidC* is controlled by an L-arabinose-inducible promoter [17]. In the presence of arabinose, both fluorescent NG chimeras show a halo-shaped signal

at the cell envelope, similar to the wild-type strain (Fig. 6a). Upon depletion of YidC, spots appeared at the cell poles for both constructs at the expense of halo formation, indicating that membrane insertion is negatively affected (Fig. 6a). Western blotting confirmed that YidC was depleted in cells grown without arabinose (Fig. 6b). The precursor form of SurA did not accumulate under the depletion conditions used, confirming that the Sec-translocon was not inactivated and over-depletion had not occurred (Fig. 6b). The cellular distribution of spots showed a distinct pattern, with the large majority of spots localized at the cell poles and a minority of spots located at mid cell (Fig. S3e and f).

To evaluate the role of the Sec-translocon, we used a strain in which *secE* is under control of an arabinose inducible promoter [18]. Depletion of SecE results in a rapid loss of the complete SecYE core of the translocon since SecY is degraded in the absence of SecE [19]. When grown in the presence of arabinose (non-depleted conditions), both fluorescent NG chimeras localized at the cell envelope, as expected (Fig. 6a). In SecE-depleted cells, both constructs still form a halo-shaped signal at the cell envelope, but small spots at the cell poles and within the cells appear, indicating moderate accumulation of mislocalized protein (Fig. 6a). Depletion of SecYE was verified by accumulation of unprocessed SurA

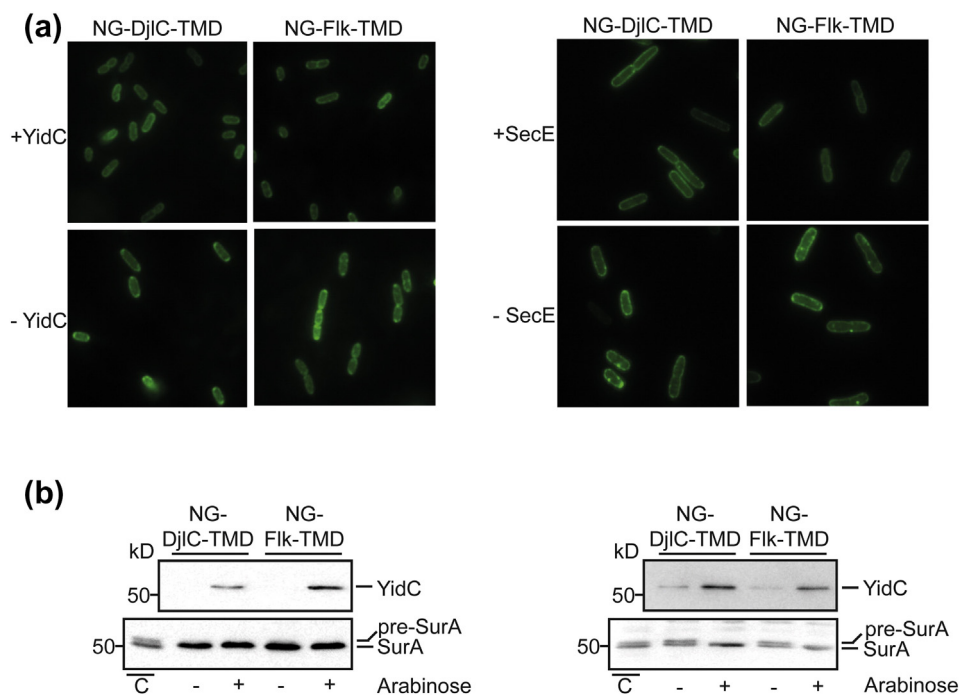


Fig. 6. YidC is required for efficient localization of NG-TMD chimeras. (a) NG-DjlC-TMD and NG-Flk-TMD were expressed under depleted and non-depleted conditions conditional for the expression of YidC and SecE. Cells were fixed with formaldehyde and analyzed by fluorescence microscopy. (b) The depletion of YidC verified by Western blotting of whole cell samples using specific YidC antibodies. SurA antibodies were used as over-depletion controls for the conditional YidC strain and as depletion controls for the conditional SecE strain. The pre-SurA control sample was obtained as described in Fig. 4.

(Fig. 6b). Of note, the level of YidC was significantly reduced even upon the short growth in the absence of L-arabinose (Fig. 6b). It is well possible that the slight aggregation of the NG chimeras under these conditions is due to this secondary effect of SecE depletion rather than reflecting a moderate dependency on the Sec-translocase *per se*.

For all strains and conditions, the expression of the chimeric constructs was verified by Western blotting (Fig. S1). In general, expression levels were similar except for NG-DjlC-TMD that was less well expressed in YidC or DnaJ/K-depleted conditions perhaps due to quality control responses.

NG-DjlC-TMD and Flk crosslink to Ffh and YidC *in vivo*

To further investigate the role of known targeting factors in TAMP biogenesis, we used site-directed photo-crosslinking to probe interactions of their TMDs *in vivo*. Incorporation of the unnatural photo-reactive amino acid *para*-benzoylphenylalanine (p-Bpa) at amber stop codon positions in our model TAMPs was achieved by co-expression of a cognate aminoacyl-tRNA synthetase/suppressor tRNA combination from the pEVOL vector [20]. We reasoned that overexpression of the p-Bpa-containing TAMPs would saturate the TAMP targeting and insertion machinery, increasing the chance to trap and identify intermediate complexes by photo-crosslinking. Since the interaction with targeting/insertion factors will most likely occur in the TMD, we created the His-tagged amber mutant NG-DjlC-TMD*271 in which the p-Bpa is incorporated at position 271 in the center of the TMD. As control, NG-DjlC-TMD*140 was created, which contains the incorporated p-Bpa at position 140 within the NG domain. After overexpression, half of the cells were subjected to UV light to induce crosslinking and the other half were kept in the dark to serve as non-irradiated control. The His-tagged p-Bpa mutants were purified from detergent-solubilized membranes, separated by SDS-PAGE and analyzed by mass spectrometry. The proteins in the analyzed samples were quantified using the exponentially modified protein abundance index [21]. Using biological triplicates for analysis, we considered known chaperones, targeting and insertion factors that were enriched at least twofold in UV-crosslinked samples as significant interaction partners.

By this criterion, NG-DjlC-TMD*271 interacted with Ffh, TF, YajC and YidC (Fig. 7a). Importantly, NG-DjlC-TMD*140 also significantly crosslinked to TF, consistent with its more generic role in protein folding and homeostasis [22]. In contrast, crosslinking from this position to YidC, Ffh and YajC was not or only barely detectable (Fig. 7a). To investigate whether the inferred interactions with the TMD are generic, we subjected Flk (without the NG fusion) to the same crosslinking procedure from three positions in and

near the TMD. The amber mutants Flk*322 and Flk*327 contain the stop codon within the TMD, whereas Flk*307 contains the stop codon in the flanking region 6 residues upstream of the TMD. Ffh, TF, YajC and YidC were strongly crosslinked from all three positions (Fig. 7b). Given the short reach and site-specific nature of the photo-crosslinking approach used, it is noteworthy that the absence of crosslinking to DnaJ/K does not necessarily reflect the absence of interaction. The interaction with the TAMPs could occur at a different position than analyzed in our site-directed crosslinking approach or be too short-lived to be trapped in a transient intermediate complex. Combined, the crosslink data suggest that the TMDs of both DjlC and Flk interact with the same targeting/insertion factors. The results further indicate that Ffh and YidC fulfill a generic role in the targeting/insertion of TAMPs through a direct interaction with the TMD consistent with the effect of depletion of these factors described above. The TMD-independent crosslinking of the polypeptides to TF is probably due to its general chaperone activity and promiscuous binding to newly synthesized proteins, including membrane proteins [23]. The raw mass spectrometry data are available in the supplemental information (Table S1).

Since we observed crosslinking to TF and YajC from both NG-DjlC-TMD and wild-type Flk, we investigated their potential function in the targeting/insertion of our two model NG chimeras using fluorescence microscopy in conditional/knockout strains. When expressed in a TF knockout strain and its isogenic wild-type (MC4100), both NG chimeras display a halo-shaped signal at the cell envelope, indicating that targeting proceeds unaffected (Fig. 8a). Western blotting confirmed the absence of TF in the knockout strain (Fig. 8b).

The crosslinking to YajC is interesting as it is part of the SecYEG–SecDF–YajC–YidC holo-translocon [24] and thus potentially contributing to the insertion of TAMPs. To evaluate the role of YajC, we created a YajC knockout derivative of strain MC4100. Expression of the NG chimeras did not show any difference in localization between the YajC knockout and its parental strain (Fig. 8a), indicating that YajC is not required for TAMP localization despite its proximity to the TMD upon overexpression. Since the *yajC* gene is located in an operon together with *secD* and *secE*, and YajC forms a heterotrimer with SecDF [25], we reasoned that the proximity of YajC could reflect a role of SecDF in membrane insertion of the model TAMPs. To test this, we expressed the NG chimeras in the arabinose-dependent SecDF–YajC depletion strain JP352 [26]. Both constructs showed halo-formation at the cell envelope irrespective of the presence or absence of SecDF–YajC (Fig. 8a), indicating that this accessory Sec complex is not required for membrane insertion. The successful depletion of SecDF–YajC was confirmed by Western blotting to detect SecD and by showing the accumulation of unprocessed SurA (Fig. 8b).

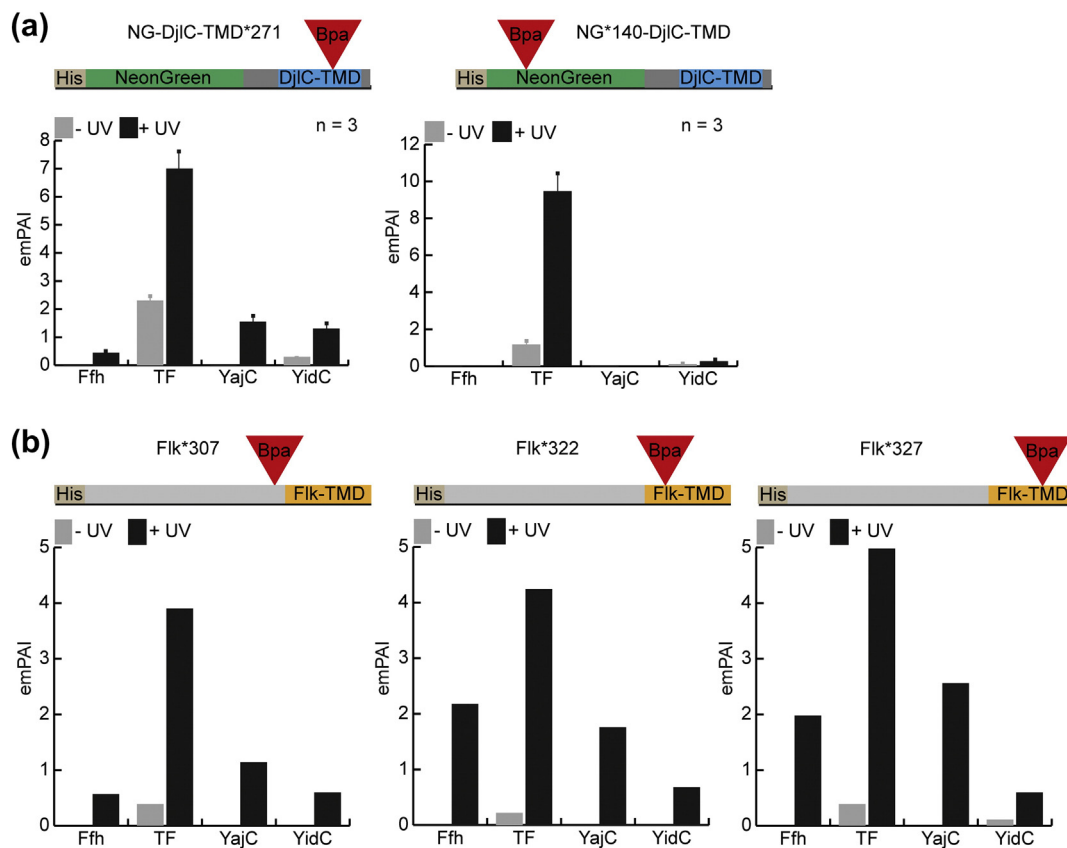


Fig. 7. *In vivo* site-directed photo-crosslinking of model TAMPs. *P*-benzoyl-phenylalanine substitution mutants of NG-DjlC-TMD and Flk were overexpressed in *E. coli* BL21(DE3) and subjected to UV light (+UV) or kept in the dark as non-irradiated control (–UV). The proteins were purified under native conditions and analyzed by mass spectrometry. The abundance of proteins in the analyzed samples was determined using the exponentially modified protein abundance index.

Discussion

Based on a prediction algorithm [3], the co-chaperone DjlC and flagellar transcriptional regulator Flk were selected as model proteins to study the mechanism of TAMP biogenesis in *E. coli*. Of note, the predicted Flk TMD is much more hydrophobic than the DjlC TMD, a feature that was shown to correlate with SRP-mediated targeting of yeast and mammalian TAMPs [2]. DjlC has a DnaJ-like domain that probably acts as a co-chaperone for the DnaK homolog HscC perhaps recruiting this chaperone for folding of membrane associated proteins [5]. Interestingly, J-domain proteins are often localized to the cytosolic face of membranes, sometimes but not always through tail-anchoring [3]. Although the hydrophobicity of the DjlC TMD is relatively moderate, we show that it is able to direct and anchor the fluorescent protein NG efficiently to the membrane. The disperse membrane distribution further suggests that the DjlC anchor is suitable as a general tag for membrane association. Notably, DjlC has a strong tendency to aggregate or associate with other proteins when expressed without its TMD. The variable size of

the aggregates formed and their resistance to various dissolving agents made it impossible to obtain reliable localization data through classical fractionation by differential centrifugation. In fact, it is difficult to exclude artifacts of this type for any mislocalized TAMP or “classical” membrane protein.

The *E. coli* flagellar regulatory protein Flk has an extremely hydrophobic TMD. Consistent with a previous study on Flk from *Salmonella typhimurium* [6], we find that it co-localizes with membranes upon fractionation. However, in contrast with this study and also different from DjlC, removal of the TMD rendered Flk completely soluble, indicating that the TMD is required for Flk localization. Similar to the DjlC TMD, the Flk TMD sequence was capable of directing NG to the membrane, albeit in a more punctate pattern, perhaps due to an interaction between the hydrophobic TMDs in the lipid bilayer.

To investigate the role of known targeting and insertion factors in the biogenesis of TAMPs, we expressed the fluorescent NG-TMD chimeras in strains conditional for the expression of these factors and determined their localization by fluorescence microscopy. Depletion of Ffh, the protein subunit of the *E. coli*

SRP and its receptor FtsY, strongly affected the membrane localization of NG-Flk-TMD, resulting in big fluorescent spots in the cells that probably represent aggregated mislocalized proteins. Analysis of the positions of the spots of NG-Flk-TMD within cells depleted of Ffh and FtsY showed a very similar and disperse distribution, showing the importance of both factors for proper targeting of NG-Flk-TMD. The effect of Ffh depletion on NG-DjC-TMD localization was similar but more moderate perhaps related to the relatively low hydrophobicity of its TMD. Strikingly, little effect on NG-DjC-TMD localization was observed in cells depleted of FtsY. Since it was shown that SRP alone is sufficient for targeting of the membrane protein TatC to YidC-containing proteoliposomes [27], it appears possible that NG-DjC-TMD can also be successfully targeted to the membrane without FtsY. Consistent with the effect of Ffh depletion, the TMD of both NG chimeras was shown to crosslink to Ffh upon overexpression. Together, the data suggest an important role of SRP-mediated targeting in TAMP localization in particular for those with a relatively hydrophobic TMD, reminiscent of TAMPs in eukaryotes [7].

Expression of both fluorescent NG chimeras in a DnaJ/K knockout strain generated a more polar spotty pattern at the expense of signal at the cell envelope. Since DnaK is a general *E. coli* chaperone with a substrate range of over 600 proteins of which ~80% are cytosolic [28], we reasoned that DnaJ/K might be

needed for proper folding of the NG domain of the chimeras, rather than being required for insertion competence of its C-terminal TMD. However, expression of NG alone in the absence of DnaJ/K did not show any fluorescent spots that indicate aggregation. In contrast, two NG chimeras consisting of NG fused to full-length DjC or Flk expressed in the absence of DnaJ/K, still generated spotty aggregates. Apparently, the role of DnaJ/K is related to the TMD rather than the fused NG domain. A role of DnaK in the biogenesis of TAMPs would correspond with a role of Hsp70 in the biogenesis of eukaryotic TAMPs [15]. On the other hand, only a subset of eukaryotic TAMPs with a mildly hydrophobic TMD appear to employ Hsp70 for their biogenesis [2], whereas our findings indicate that in *E. coli* TAMPs with strongly hydrophobic TMDs (like Flk) also require DnaK for membrane localization. Although further analysis is required to reveal a direct function of DnaK in TAMP biogenesis, our data suggest an important, and perhaps generic, role for DnaK in maintaining bacterial TAMPs in a soluble and insertion competent form after their release from the ribosome.

Interestingly, a recent study showed that co-overexpression of membrane proteins with DjIA, a membrane-anchored co-chaperone of DnaK, enhances the production of membrane proteins [29], possibly by recruiting DnaK and its client proteins to the inner membrane. By extrapolation, one might speculate that DjIA could play a role in the targeting of

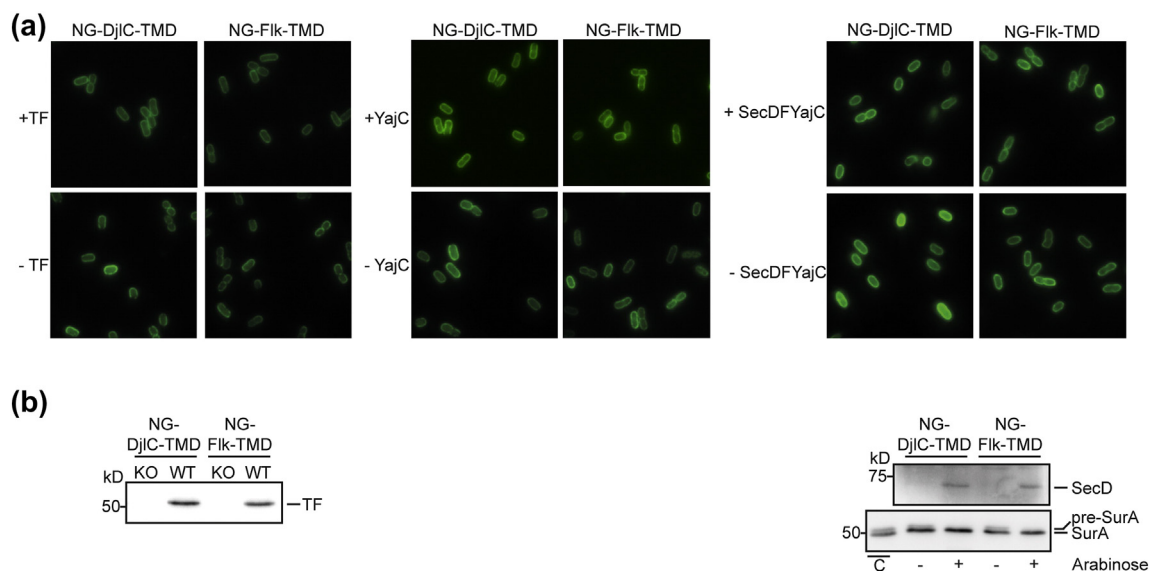


Fig. 8. TF and SecDF–YajC are not required for efficient localization of NG-TMD chimeras. (a) NG-DjC-TMD and NG-Flk-TMD were expressed in TF and YajC knockout strains and their isogenic wild-type strain MC4100, as well as under depleted and non-depleted conditions in a strain conditional for the expression of SecDF–YajC. Cells were fixed with formaldehyde and analyzed by fluorescence microscopy. (b) The depletion of SecDF was confirmed by Western blot analysis of whole cells with SecD antibodies and by the accumulation of pre-SurA. The pre-SurA control sample was obtained as described in Fig. 4. The absence and presence of TF in the knockout (KO) and wild-type (WT) strains were verified using specific TF antibodies. The absence of YajC was verified by confirming the correct insertion of the kanamycin cassette into the *yajC* gene using colony PCR (see Materials and Methods).

TAMPs. However, expression of both NG-DjlC-TMD and NG-Flk-TMD in a DjlA knockout strain did not affect localization of either protein, indicating that anchoring of DnaK to its membrane receptor is not required for the targeting of both NG chimeras.

Whether SRP or DnaK acts sequentially or simultaneously on different regions of the TAMPs remains to be determined. In any case, the strong effects observed upon depletion/deletion of either Ffh or DnaK argue against the functional redundancy in targeting pathways observed in eukaryotes [2]. Studies on the biogenesis of SciP, a single spanning inner-membrane protein with a C-terminal TMD, suggested an SRP-dependent targeting mechanism, whereas DnaJ/K proved dispensable for its targeting and insertion [30]. Interestingly, in the same study, two hydrophobic stretches were identified in the N-terminal region of SciP that were shown to be both required and sufficient for successful membrane targeting. Since TAMPs are characterized by the lack of an N-terminal targeting sequence, SciP cannot be considered a *bona fide* TAMP and may well follow a different pathway for membrane targeting and insertion.

In addition to Ffh, site-directed photo-crosslinking from the TMD of the NG chimeras also revealed an interaction with the cytoplasmic chaperone TF. However, in contrast to Ffh, crosslinking to TF was not restricted to the TMD and its association most likely reflects the general chaperone activity and promiscuous substrate recognition of TF [22]. The precise interplay between SRP and TF at the TMD of the TAMPs remains to be investigated, but TF is clearly not required for efficient membrane targeting.

YidC is known to be involved in the insertion of Sec-dependent and -independent membrane proteins [31], and we find it also necessary for the efficient membrane localization of both NG chimeras employed in this study. Furthermore, the proximity of YidC to the TMD of both DjlC and Flk was shown by *in vivo* site-directed photo-crosslinking. SciP fused to GFP was also shown to localize in a YidC-dependent way [30] and accumulated upon YidC depletion at the cell poles, reminiscent of the behavior of the fluorescent NG chimeras used in this study. A study on seven very small membrane proteins (<50 amino acids) that qualify as TAMPs revealed that four of the tested proteins required YidC for efficient co-fractionation with membranes [32]. Comparable results based on subcellular fractionation have also been obtained for TssL (SciP) [33]. However, the localization of these proteins was determined by biochemical approaches, which have to be evaluated with caution.

Although not detected as crosslinking partner for the TMD of the NG chimeras, we also investigated the role of the Sec-translocon using a strain conditional for the expression of *secE* [18]. In cells depleted of SecE, both NG chimeras were still localized at the membrane but small dots, probably representing mislocalized and aggregated proteins, started to accumulate.

Since a significant reduction in the amount of the Sec-dependent YidC was observed in the depleted cells, it is entirely possible that the observed effects are secondary to the low levels of YidC that are maintained under these conditions. Earlier work showed minor effects in the insertion of SciP and the very small inner-membrane proteins AtpE, LebP, YbgT and YkgR upon SecE depletion [30,32], but YidC levels were not evaluated in these studies. It should be noted that YidC depletion has a much stronger effect on the Sec-independent inner-membrane proteins than on Sec-dependent proteins [34], suggesting that the NG chimeras used in this study employ YidC for their insertion in a way that is independent of the Sec-translocon.

Finally, YajC was identified as a crosslinking partner of the TAMP TMDs. Interestingly, although the exact function of YajC has not been elucidated, it is part of the auxiliary SecDF–YajC complex [35]. However, deletion of YajC or depletion of SecDF had no discernable effect of localization of the NG chimeras. Possibly, YajC is close to YidC where membrane insertion occurs but is not required for the insertion process *per se*, although we cannot exclude that it influences the kinetics or fidelity of the process. Intriguingly, although its function remains enigmatic, YajC has been found in complex with other inner-membrane proteins [36] including YidC (de Gier, unpublished data).

In conclusion, we show that the TMDs of two *E. coli* model TAMPs are both required and sufficient for membrane localization. SRP, FtsY and DnaJ/K are, to different extents, required for their membrane targeting and to prevent aggregation, whereas YidC plays an important role in membrane insertion that appears to occur largely independent of the Sec-translocon. Future studies will focus on the way in which the SRP and YidC act in the post-translational insertion of TAMPs as compared to the co-translational insertion of “classical” inner-membrane proteins with similar requirements.

Materials and Methods

Strains and growth conditions

E. coli TOP10F' was used as cloning strain as well as expression strain for cell fractionation and fluorescence microscopy in wild-type conditions. *E. coli* BL21(DE3) was used for the *in vivo* site-directed photo-crosslinking. The YidC depletion strain MK6s [17], the Ffh depletion strain HDB51 [12], the FtsY depletion strain IY26 (provided by Eitan Bibi) and the SecDF–YajC depletion strain JP352 [26] were grown in sodium phosphate-buffered LB supplemented with 0.4% L-arabinose and 0.2% D-glucose (non-depleting conditions), or only with 0.2% glucose (depleting

conditions). For depletion of Ffh, FtsY and SecDF–YajC, overnight cultures were diluted to OD₆₆₀ 0.01 in medium with or without arabinose and grown for 3 h (Ffh, FtsY) or 4 h (SecDF–YajC). For YidC depletion, overnight cultures were diluted to OD₆₆₀ 0.05 and regrown to OD₆₆₀ 0.2 and then split into cultures with or without arabinose and grown for 3 h. The SecE depletion strain CM124 was grown as described earlier [18], in sodium phosphate-buffered LB without thiamine. The depletion time for SecE was 1 h. The knockout strains MC4100Δ*tig* [37], MC4100Δ*dnaJK* (grown at 30 °C) [38], MC4100Δ*djlA* [39] and MC4100Δ*yajC* and their isogenic wild-type MC4100 were grown in LB.

Plasmid construction

Plasmid pSE(p15a) FLAG-DjlC spR was created by replacing the ColE1 origin and the ampicillin resistance marker from the original pSE380 FLAG-DjlC (gift from Pierre Genevaux). The p15a origin and the chloramphenicol resistance marker from the vector pEH3 (p15a) were PCR-amplified using the forward primer 5'-GGCAGCCGCATATGGGGTCTGAATTTGCTTTCG-3' and the reverse primer 5'-CCAAAGGATCCAGAAGGCCATCCTGACGGATGGCCTTTGAATTCGTGCGTAACGGC-3'. The PCR fragment was cloned into pSE380 FLAG-DjlC, resulting in the chloramphenicol resistant pSE(p15a) FLAG-DjlC cmR. The spectinomycin resistance marker of pCL1920 was PCR-amplified using the forward primer 5'-CCATTCGTGACCTTAGTGATCTAAC-3' and the reverse primer 5'-CCATTCATATGTTCTGCTGTGCCAA-3' and cloned into pSE(p15a) FLAG-DjlC cmR by NdeI and Sall, creating pSE(p15a) FLAG-DjlC spR. A kanamycin-resistant derivative, pSE(p15a) FLAG-DjlC spR, was created similarly by amplifying the kanamycin resistance marker of pEH1 using the forward primer 5'-CAACATATGTTTACTTTGCAGGCTTC-3 and the reverse primer 5'-CAAGTCGACGCGAGTCTTCTTGAGCAG-3'. FLAG-DjlCΔTMD was created by PCR using the forward primer 5'-CAACCATGGACTACAAAGACGACGAC-3' and the reverse primer 5'-CAAGGATCCTCAGCTCTGTGGT TTTTC-3' and cloning into pSE(p15a) spR via NcoI and BamHI. FLAG-Flk and FLAG-FlkΔTMD were created similarly using the forward primer 5'-CCACATGGACTACAAAGACGATGACGACAAGATGATACAACCTATTTCC-3' and the reverse primers 5'-TTCGGGATCCTTAACGAACCAGCCAG-3' (full-length) or 5'-CAAGGATCCTCAATTTTGCAACGTGTC-3' (ΔTMD). His-NG was PCR-amplified with the forward primer 5'-CAACCATGGGACACCACCACCA CCACATATGGTGAGCAAGGGCGAG-3' and the reverse primer 5'-CAAGGATCCTTAGAATTCCTTG TACAGCTC-3'. The fluorescent tail-anchored chimeras NG-DjlC-TMD and NG-Flk-TMD consist of an N-terminally His-tagged NG fused to the TMD of either DjlC or Flk, using the 20 residues upstream of the

TMD as linker sequence. These constructs were created using overlap extension PCR. NG was PCR-amplified with the above-mentioned forward primer and with the reverse primer 5'-GAATTCCTTGTA CAGCTCGTCCATGCC-3'. The gene fragments for the TMDs of DjlC and Flk were PCR-amplified using the forward primers 5'-TACAAGGAATTCGATGAGC-CAACACCTGAA-3' (DjlC) and 5'-TACAAGGAAT CCCGCGTGTATGCAACCG-3' (Flk) and the reverse primers 5'-CAAGGATCCTCAGCTAAACAAC ATCGC-3' (DjlC) and 5'-TTCGGGATCCTTAAC- GAACCAGCCAG-3' (Flk). N-terminally FLAG-tagged and C-terminally Opsin-tagged NG chimeras were constructed using the forward primer 5'-CAATT CCATGGGAGATTACAAAGACGATGACGACAA GATGGTGAGCAAGGGCGAG-3' and the reverse primers 5'-CAATTGGATCCTCAGCCCGTCTTGT G G A G A A A G G C A C G T A G A A G T T TGGGCCGCTGCCGCTCCTCCGCTAAACAA CATCGC-3' (DjlC) and 5'-CAATTGGATCCTTAGCC CGTCTTGTGGAGAAAGGCACGTAGAAGTTTG GGCCGCTGCCGCTCCTCCACGAACCAGCCA GAC-3' (Flk). Amber mutants of NG-DjlC-TMD and wild-type Flk were created using overlap extension PCR.

Construction of MC4100Δ*yajC* strain

The MC4100Δ*yajC* strain was engineered using the λ-Red recombineering-based approach developed by Datsenko and Wanner [40]. In short, a kanamycin cassette with flanking regions homologous to the up- and down-stream regions of the *yajC* gene was generated by PCR using the pKD13 plasmid as a template and the forward primer 5'-GAGAAGTAC CACCTTTGAACGTTGATTAATTAATAATG AGGGAAATTTAATTCCGGGGATCCGTCGACC-3' and reverse primer 5'-GAGAAGTACCACCTTTGAA CGTTGATTAATTAATAATGAGGGAAATTTAA TTCCGGGGATCCGTCGACC-3'. Subsequently, the PCR reaction was treated with *DpnI* (NEB cutsmart) to get rid of the template DNA. The PCR reaction was loaded on an agarose gel to verify the molecular weight of the generated PCR product and it was subsequently purified from the gel using the QIAGEN gel extraction kit. The purified PCR product was electroporated into *E. coli* MC4100 cells harboring pKD46 that were cultured in standard LB medium in the presence of 0.2% arabinose at 30 °C. After verifying the correct insertion of the kanamycin cassette into the *yajC* gene using colony PCR and the primers *yajC*-up and *yajC*-down, *yajC*:Km^R was transferred from MC4100*yajC*:Km^R/pKD46 to wild-type MC4100 by means of P1-transduction [41] to generate MC4100*yajC*:Km^R. MC4100*yajC*:Km^R was transformed with pCP20 to flip out the Km^R-cassette. Flipping out of the Km^R-cassette was checked by means of colony PCR using forward primer 5'-GGTTA AGCGCCACGACGTATTTG-3' and the reverse primer

5'-GATCCTGAGTGTGATTGCTACACC-3'. Not only the size, but also the sequence of the PCR product was checked. Finally, it was verified that the prolonged cultivation of MC4100DyajC at 37 °C had resulted in curing it from pCP20.

Cell fractionation

E. coli TOP10F' harboring derivatives of pSE(p15a) were grown to OD₆₆₀ 0.3 and protein expression was induced for 20 min with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested for 10 min at 4600g and 4 °C, resuspended in PBS and lysed using the OneShot cell disruptor (Constant Systems Ltd) at 1.82 kBar. Debris and unlysed cells were spun down as above (low-speed pellet) and the supernatant was subjected to ultracentrifugation for 90 min at 347,000g to collect the membranes.

Membrane extractions

Crude membranes of *E. coli* TOP10F' cells expressing TAMP derivatives were isolated as described in the cell fractionation. The crude membranes were resuspended in PBS and split into 3 fractions. Each fraction was mixed with PBS (control), 0.2 M Na₂CO₃ or 2% DDM. For urea extraction, crude membranes were resuspended in 20 mM Tris (pH 8) and 150 mM NaCl with urea concentrations of 4, 6 or 8 M. PBS and Na₂CO₃ samples were incubated on ice for 30 min. The DDM and urea samples were incubated on a turntable at 4 °C for 1 h. Samples were subjected to ultracentrifugation for 45 min at 190,000g and 4 °C. Pellet fractions were directly resuspended in SDS sample buffer. Supernatant fractions were TCA-precipitated and resuspended in SDS sample buffer.

Sucrose gradient centrifugation

E. coli TOP10F' harboring pSE(p15a) FLAG-DjlC or pSE(p15a) FLAG-DjlCΔTMD were grown in a volume of 500 ml to OD₆₆₀ 0.3, and protein expression was induced for 20 min with 0.5 mM IPTG. Cells were harvested for 10 min at 4600g and 4 °C, washed with 100 ml buffer K [50 mM TEA, 250 mM sucrose, 1 mM EDTA, 1 mM DTT (pH 7.5)], harvested again as above and resuspended in 6 ml buffer K supplemented with DNase. Crude membranes were isolated as above and resuspended in buffer K, applied to a 30%–50% sucrose gradient [50 mM TEA, 1 mM EDTA, 1 mM DTT, 30%–50% sucrose (pH 7.5)] and spun for 20 h at 94,000g (TST 14.41 rotor) and at 4 °C. Fractions were taken from the top of the gradient to bottom, TCA-precipitated and analyzed by Western blotting.

Fluorescence microscopy

Cells were fixed with 2.8% formaldehyde for 10 min, washed twice with PBS and stored at 4 °C. For

analysis, 0.03 OD₆₆₀ units of fixed cells were applied to poly-L-lysine-coated glass slides (Sigma-Aldrich) and microscopy was performed on an Olympus BX 60 microscope.

ProtK accessibility in spheroplasts

E. coli MC4100 cells expressing NG-DjlC-TMD and NG-Flk-TMD with an N-terminal FLAG-tag and C-terminal Opsin-tag were harvested for 2 min at 16,000g and resuspended in 100 mM Tris (pH 8) and 1 M sucrose. After addition of 1 mM EDTA (pH 8) and 5 µg/ml lysozyme, cells were incubated at RT. After 15 min, the spheroplasts formation was checked by phase contrast microscopy and stopped by adding 20 mM MgCl₂. The spheroplasts were harvested for 2 min at 6000g and 4 °C; resuspended in 100 mM Tris (pH 8), 250 mM sucrose and 20 mM MgCl₂; and kept on ice. Spheroplasts were mixed with either buffer, 50 µg/ml ProtK or 50 µg/ml ProtK and 1% TritonX-100 and incubated for 20 min at 25 °C. Subsequently, ProtK was inactivated by addition of 100 mM PMSF and incubation for 10 min at 4 °C. The samples were analyzed by SDS-PAGE and Western blotting.

Site-specific *in vivo* UV-crosslinking

E. coli BL21(DE3) cells harboring pEVOL and one of the pET16b His-NG-NG-DjlC-TMD or pET16b His-Flk amber mutant variants were grown in LB supplemented with the appropriate antibiotics. At OD₆₆₀ 0.5, 0.2% L-arabinose for the induction of pEVOL and 0.5 mM p-Bpa (Bachem AG) were added. After continued growth for 30 min, the expression of the TAMP amber mutants was induced with 1 mM IPTG and the cells were grown for additional 30 min. After induction, UV-crosslinking was performed as described earlier [42]. After crosslinking, cells were lysed using the One Shot cell disruptor at 1.82 kBar and cell debris/unlysed cells were spun down for 10 min at 4600g at 4 °C. The crude membranes were isolated by ultracentrifugation for 1 h at 293,100g and 4 °C in a MLA80 rotor. The crude membranes were resuspended in buffer A supplemented with cComplete™ EDTA-free Protease Inhibitor Cocktail (Roche). DDM was added to 2% and membranes were solubilized overnight at 4 °C on a turning wheel. Unsolubilized material was spun down for 40 min at 300,000g at 15 °C. The supernatant was diluted to 1% DDM and applied to His SpinTrap TALON columns (GE Healthcare). The purification was performed according to the manufacture's protocol using washing buffer W (50 mM Na₂HPO₄, 300 mM NaCl, 10% glycerol, 0.1% DDM, 20 mM imidazole) and elution buffer E (50 mM Na₂HPO₄, 300 mM NaCl, 10% glycerol, 0.1% DDM, 400 mM imidazole). Elution fractions were mixed with SDS sample buffer.

Mass spectrometric analysis

Protein lanes from Coomassie-stained SDS-PAGE gels were excised and prepared for MS/MS analysis as described earlier [43]. Data analysis was performed using the Mascot Server (Matrix Science, London, UK).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2017.12.004>.

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Abbreviations used:

TAMP, tail-anchored membrane protein; TMD, trans-membrane domain; SRP, signal recognition particle; NG, mNeonGreen; ProtK, Proteinase K; GRAVY, Grand Total Average of Hydropathy; DDM, *N*-dodecyl- β -D-maltoside; p-Bpa, *para*-benzoylphenylalanine.

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