Role for *Escherichia coli* YidD in Membrane Protein Insertion†‡

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YidC has an essential but poorly defined function in membrane protein insertion and folding in bacteria. The yidC gene is located in a gene cluster that is highly conserved in Gram-negative bacteria, the gene order being *rpmH, rpmA, yidD, yidC*, and *trmE*. Here, we show that *Escherichia coli yidD*, which overlaps with *rpmA* and is only 2 bp upstream of *yidC*, is expressed and localizes to the inner membrane, probably through an amphipathic helix. Inactivation of *yidD* had no discernible effect on cell growth and viability. However, compared to control cells, ∆yidD cells were affected in the insertion and processing of three YidC-dependent inner membrane proteins. Furthermore, in vitro cross-linking showed that YidD is in proximity of a nascent inner membrane protein during its localization in the Sec-YidC translocon, suggesting that YidD might be involved in the insertion process.

Membrane proteins function in critical cellular processes such as energy transduction, transport of molecules, cell communication, and cell division. Upon membrane targeting and insertion, the membrane proteins need to fold and often to assemble into oligomeric structures for proper functioning (31, 39). The Sec translocon plays an important role in the insertion of most membrane proteins into the inner membrane (IM) of *Escherichia coli* (12). It consists of the conserved heterotrimeric complex SecYEG and the accessory components SecDF-YajC, whose function is not entirely clear although a recent study suggested that SecDF functions as a proton motive force (PMF)-dependent chaperone for preproteins (41). Cross-linking and pulldown experiments have identified YidC as another Sec-associated factor (36). For a subset of inner membrane proteins (IMPs) such as Lep, FtsQ, and MtiA, YidC has been shown to associate with the transmembrane (TM) segments of nascent protein chains upon their lateral exit from the Sec translocon (7, 50). However, depletion of YidC in vivo only slightly affects the levels of these Sec-dependent IMPS, indicating that it is not essential for insertion. YidC can also act upstream of the Sec translocon, as demonstrated for the lipoprotein CyoA (subunit II of the cytochrome *bo* oxidase) (4, 13, 45). YidC is sufficient to catalyze insertion of the N-terminal domain of CyoA while translocation of the large C-terminal domain requires the Sec translocon. For another subset of IMPs, YidC alone is sufficient for insertion and assembly of the complete protein into the IM. Substrates of this YidC-only pathway include small coat proteins of the M13 and Pf3 phages, the subunits a and c of the F₁Fₐ ATPase (Fₐ,a and Fₐ,c), and the subunit K of the NADH dehydrogenase complex (34, 56). YidC has also been copurified with the membrane protease FtsH and its modulator proteins HflK/HflC, suggesting an early, linked role in the quality control of membrane proteins (44).

The coding sequence for the essential *yidC* gene is located in a cluster of genes involved in protein synthesis and membrane targeting that is highly conserved in Gram-negative bacteria, with the gene order being *rpmH, rpmA, yidD, yidC*, and *trmE* (see Fig. S1 in the supplemental material). In general, such a conserved genetic organization suggests coordinated gene expression and a related function. Indeed, three promoters have been located upstream of *rpmH*, one of which generates a polycistronic mRNA (21). *rpmH* codes for the ribosomal protein L34, a small basic protein of the large ribosomal subunit. L34 has been cross-linked to L23 that participates in a docking site (48) for the signal recognition particle (SRP), SecA, and the chaperone trigger factor near the nascent chain exit region. *rpmA* encodes the protein component of RNase P, an endoribonuclease that processes tRNA precursor molecules but also the 4.5S RNA component of the SRP (2). *trmE* encodes an Era-type GTPase that functions in a tRNA modification pathway and has been implicated in the control of the glutamate-dependent acid resistance system (20). Interestingly, the growth defect of YidC-depleted cells can be alleviated by overexpression of GadX and GadY, transcriptional activators that are also involved in regulation of this acid resistance mechanism (54).

In comparison to YidC, very little is known about the expression and function of *yidD*, but its presence and location in the gene cluster are highly conserved. We set out to characterize *yidD* because of our interest in inner membrane protein biogenesis and the functional relation of the remainder of the gene cluster in that process. The *yidD* gene is sandwiched between *rpmA* (37-bp overlap) and *yidC* (2-bp spacing) and is likely to contain an internal promoter for *yidC* (3). In the
present study, we have verified the expression of yidD in E. coli. YidD was found to be associated with the IM via a putative amphiphatic α-helix in its N-terminal region. Although YidD is not essential for growth, our data indicate that YidD is required for efficient insertion and maturation of YidC-dependent IMPs. Furthermore, using an in vitro sulfhydryl cross-linking approach, YidD was found in close proximity to a short nascent IMP, suggesting a direct role for YidD during IMP biogenesis.

MATERIALS AND METHODS

Enzymes and material. Restriction endonucleases and other DNA-modifying enzymes were obtained from Roche and Invitrogen. Bis-maleimidoethane (BMOE) was from Pierce. All other chemicals were supplied by Sigma. Antiseras against the His tag and the hemagglutinin (HA) tag were purchased from Roche and Sigma, respectively. Antiseras against YidC, PspA, Lep, Foc, and TolC have been described previously (27, 47).

Strains, plasmids, and primers. All strains, plasmids, and primers are listed in Table 1. All strains were grown in Luria-Bertani (LB) medium with appropriate antibiotics, unless stated otherwise. E. coli Top10F’ (Invitrogen) was used for cloning and maintenance of plasmid constructs. Strain MC4100-A was routinely used for expression and preparation of inner membrane vesicles (IMVs) and translation extracts. The MC4100-A strain (23) was a generous gift of Frank Sargent (University of East Anglia, Norwich, United Kingdom). The ΔyidD strain was constructed according to Datsenko and Wanner (8). The primers YidD_Ecoli_swap_H1 and YidD_Ecoli_swap_H2 were used to amplify the yidD ORF with 5’ terminal sequences. Proper inactivation of yidD was verified by PCR.

Plasmid pTL61T (30) served as a template to construct the lacZ transcriptional fusion pTL61T-Nco-yidDprom. First, an NcoI site was introduced with the primers pTL61T-NcoFw and pTL61T-NcoRv, which kanamycin-resistant colonies were selected. Proper inactivation of yidD was verified by PCR.

### Table 1. Strains, plasmids, and primers

<table>
<thead>
<tr>
<th>Strain, plasmid, or primer</th>
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<tr>
<td>E. coli strains</td>
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<td>Top10F’</td>
<td>F’ lacZ Δ(mrr-hsdRMS-mcrBC) Δ88locZΔM15 ΔlacXΔ74 supG recA1 araD139 Δ(ara-lev)7697 galE15 galK16 rpsL(St) endA1 λ</td>
<td>Invitrogen</td>
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<td>MC4100-A</td>
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<td>MC4100(DE3)</td>
<td>MC4100 with λ DE3</td>
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<tr>
<td>ΔyidD strain</td>
<td>yidD knockout in MC4100 (DE3)</td>
<td>This study</td>
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<td>Plasmids</td>
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<td>pTL61T</td>
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*YidD* was verified by PCR.
primers pTL61T-NcoFw and pTL61T-NcoR at the start of the lacZ open reading frame (ORF) in pTL61T, resulting in pTL61T-Ncol, using a QuickChange Site-Directed Mutagenesis Kit (Stratagene). Then, a ~924+/−3 region 5′ of the yidD start codon was mutated to remove an endogenous Ncol site using the primers YidDpromeinNcoFw and YidDpromeinNcoRv. This region, then, was amplified using the primers YidDpromeinFw and YidDpromeinRv to introduce an Ncol site at the start codon of YidD and cloned into the Ncol site of pTL61T-Nco, resulting in pTL61T-Ncol-YidDpromein.

Primers pEH3 (22) was used for the expression of His-YidD. The gene encoding yidD was amplified using E. coli MC4100 genomic DNA as a template with the primers YidDNterm-Xba-SD-His6Fw and YidDterm-SacIRv and cloned into Xba1/SacI-digested pEH3 to construct pEH3His-YidD, pEH3GFP-YidD (where GFP is green fluorescent protein), pEH3GFP-YidDH1, and pEH3GFP-YidDpromein as constituting sequences. The coding sequence of GFP was amplified from the pEGFP plasmid (Clontech) using the primers pEGFP Fw and pEGFP Rev and cloned into the pEGFP-N1 vector (Clontech) to produce pEGFP-YidD. The product of the PCR using the primers pEGFP Fw and pEGFP Rev while the coding sequence for YidD was amplified from MC4100 genomic DNA with the primers YidD5′ GSFS and YidD3′ stopRv. These two PCR products were used as templates for the subsequent overlapping PCR with the primers GFP5′ XbaFw and YidD3′ stopRv to yield the amplification products encoding the GFP-YidD fusion flanked by the Xba1 and SacI sites. This product was cloned into Xba1/SacI-digested pEH3 to yield pEH3GFP-YidDpromein. Similarly, to construct pEH3GFP-YidDH1 containing gfp fused to the first 81 bases of yidD, the product of the PCR using the primers pEGFP Fw and pEGFP Rev was combined with the PCR product using the primers YidD5′ GSFS and YidD3′ NtermstopRv and used as a template in an overlapping PCR with the primers GFP5′ XbaFw and YidD3′ NtermstopRv. To construct pEH3GFP-YidDpromein, the coding GFP was amplified with the primers GFP5′ XbaFw and GFP3′ SacIRv/GFP5′ XbaFw and GFP3′ SacIRv while the coding sequence for YidD was amplified from MC4100 genomic DNA with the primers YidD5′ GSFS and YidD3′ stopRv.

Protein sample analysis. Protein samples were routinely analyzed by SDS-PAGE and Western blotting. Protein quantifications were performed using ChemiDoc XRS (Bio-Rad) with Quantity One (Bio-Rad) software.

RESULTS

Genomic organization of yidD. The E. coli yidD is located in an interesting gene cluster comprising the following genes: rpmH, rpmA, yidD, yidC, and trmE (Fig. 1A). This region has been strikingly conserved among the Gram-negative bacteria (see Fig. S1 in the supplemental material) (16). Homologs of yidD are also widely spread in Gram-positive bacteria, but the genomic context in these cases is less conserved. Interestingly, yidD homologs, defined by the presence of the conserved domain of unknown function 37 (DUF37) (5), are found in all plants sequenced so far but not in yeast, Caenorhabditis elegans, or mammals. In addition, yidD is found in the genome of Haemophilus influenzae phage HP1 (17). The yidD homologs in plants contained neither a clear mitochondrial nor a chloroplast localization signal as predicted by the subcellular localization programs PSORTII (24) and TargetP (15). Nor were the yidD homologs found to colocalize with alb3 and oxal1 in the genome.

E. coli YidD has a predicted size of 9.3 kDa and a pI of 10.2. The DUF37 domain in the yidD sequence is characterized by three conserved cysteines (Fig. 1B and C). Some members of the yidD family have been annotated as hemo-
ylases, which resulted from the unpublished observation reported in GenBank L36462 that the Aeromonas hydrophila YidD homolog HlyA possesses alpha-hemolysin activity (35). However, the Aeromonas HlyA does not appear to be related to any of the known hemolysins. Secondary structure prediction indicated the presence of three α-helices in YidD (Fig. 1C). None of the three α-helices appeared sufficiently hydrophobic to serve as a TM, suggesting a cytoplasmic localization for YidD. However, a closer examination of the α-helical wheel projection of the predicted first α-helix in YidD suggested an amphipathic structure (Fig. 1D).

Transcription and translation of yidD. The Proteus mirabilis yidD open reading frame (ORF) has been shown to be expressed in E. coli by using a translational LacZ fusion (38). However, there are no reports available on the putative E. coli yidD, and we therefore verified its expression. First, we determined whether mRNA transcript coding for YidD is produced in E. coli MC4100 cells by performing reverse transcription-PCR (RT-PCR) on total RNA isolated from logarithmically growing MC4100 cells (Fig. 2A). Using primers that were specific for the yidD sequence, we could detect yidD mRNA (lane 5). A reaction with primers for the adjacent yidC gene (which is known to be expressed) served as a positive control and yielded amplification products of the expected sizes (lanes 6 and 7). To ensure that the observed RT-PCR products were not due to DNA contamination, negative controls were performed in which the reverse transcriptase was omitted from the cDNA synthesis reaction. As shown (Fig. 2A, lanes 1 to 4), no amplification products were detected under these conditions, verifying that the products generated by RT-PCR were due to the presence of transcript and not due to genomic DNA contamination. As a further positive control for the PCR amplification, genomic DNA of the strain MC4100 was used as a positive control and yielded amplification products of the expected sizes (lanes 6 and 7). To ensure that the observed RT-PCR products were not due to DNA contamination, negative controls were performed in which the reverse transcriptase was omitted from the cDNA synthesis reaction. As shown (Fig. 2A, lanes 1 to 4), no amplification products were detected under these conditions, verifying that the products generated by RT-PCR were due to the presence of transcript and not due to genomic DNA contamination. As a further positive control for the PCR amplification, genomic DNA of the strain MC4100 was used as a

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template, which resulted in PCR products of the same size as
the products in the RT-PCR amplification (Fig. 2A, lanes 9 to
12). Furthermore, a PCR product was formed (lane 8) when
primers were used that corresponded to the 5' end of
\textit{yidD} and the center of \textit{yidC}, indicating that \textit{yidD} is, at least in part,
cotranscribed with \textit{yidC}. Thus, RT-PCR analysis confirmed
that \textit{yidD} transcript is synthesized and cotranscribed with \textit{yidC}
mRNA.

The transcriptional activity of the putative promoter up-
stream of \textit{yidD} was analyzed using a \(\beta\)-galactosidase reporter

![FIG. 1. \textit{In silico} analysis of \textit{E. coli} \textit{yidD}. (A) Genomic context of the \textit{yidD} gene in \textit{E. coli}. ORFs are represented by arrows in 5' to 3' direction. (B) ClustalW alignment of selected \textit{yidD} gene family members containing the DUF31 conserved sequence. (C) Schematic representation of the predicted secondary structure of \textit{E. coli} \textit{YidD}. The boxes represent putative \(\alpha\)-helices, and the lines represent coiled regions of \textit{YidD}. (D) Helical representation of the first putative helix of \textit{E. coli} \textit{YidD} as predicted by http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html. \textit{M. tuberculosis}, \textit{Mycobacterium tuberculosis}; \textit{B. subtilis}, \textit{Bacillus subtilis}; \textit{S. aureus}, \textit{Staphylococcus aureus}; \textit{P. aeruginosa}, \textit{Pseudomonas aeruginosa}; \textit{N. meningitidis}, \textit{Neisseria meningitidis}; \textit{A. thaliana}, \textit{Arabidopsis thaliana}; \textit{H. pylori}, \textit{Helicobacter pylori}.}
system. The ~1-kb region upstream of the coding sequence of yidD was cloned into pTL61-Nco, resulting in a lacZ reporter gene that is directly fused to the start codon of yidD. Using a β-galactosidase assay, significant activity of the yidD-lacZ fusion was detected in E. coli MC4100 compared to activity in the promoterless lacZ control (Fig. 2B). Together, the expression data indicate that yidD is transcribed and translated in E. coli.

Expression and subcellular localization of YidD. To monitor the expression of yidD, the gene was cloned under lac promoter control in the expression vector pEH3 (22). A sequence encoding a His6 tag was fused to the 5' end of yidD, allowing the detection of the expressed protein. As shown in Fig. 2C (upper panel), a 14-kDa product was detected in IPTG-induced cells upon Western blotting using antiserum directed against the His6 tag. Apparently, His-YidD migrates more slowly than the predicted 9.4 kDa, which may be caused by the addition of the His tag. His-YidD expression did not affect growth, and the protein could not be detected by total Coomassie protein staining, indicating that his-yidD is not expressed at a high level (not shown). Fusion of the His6 tag-encoding sequence to the 3' end of yidD yielded only a faintly detectable product of 14 kDa upon Western blotting, suggesting that the C-terminal tag destabilized the protein (data not shown).

IPTG induced expression of His-YidD resulted in a slight increase of PspA expression (Fig. 2C, middle panel). PspA is a peripheral IM-associated stress protein that responds to impaired membrane function (26, 52). Although the biological function of PspA is far from clear, it appears to play a role in the maintenance of the PMF upon cell envelope stress. It was shown previously that YidC-depleted cells have increased levels of PspA to counteract a loss of PMF due to the impaired insertion of respiratory chain complexes (47). However, the level of YidC was not detectably changed upon overexpression of His-YidD (Fig. 2C, lower panel). Therefore, the PspA response—albeit modest—caused by His-YidD overexpression suggests an effect on membrane functioning, independent of the YidC level. The level of the cytoplasmic heat shock protein GroEL was not altered upon His-YidD expression, arguing against a more general stress phenomenon (data not shown).

The link with membrane stress was surprising because YidD is predicted to be located in the cytoplasm, considering the absence of a putative signal sequence and of hydrophobic TMs. We therefore experimentally verified the subcellular localization of YidD. Cells in logarithmic growth phase that produced His-YidD were harvested and lysed, after which total membranes were isolated by centrifugation. Interestingly, Western blot analysis of the various fractions taken during the isolation showed that His-YidD cofractionated with membranes in the high-speed pellet (Fig. 3A, lane 4). As a control for the fractionation, the samples were analyzed by Western blotting using antibodies against leader peptidase (Lep), a bona fide IMP. YidD and Lep were found in the same fraction. In these experiments, the His-YidD protein level appeared to be decreased during the fractionation procedure. For the cell debris (D) (Fig. 3A, lane 2) and soluble (S) (lane 3) and membrane

![Figure 2](http://jb.asm.org/)
The absence of an obvious signal sequence would suggest that His-YidD is associated with the IM rather than the OM. To confirm this, crude membranes from His-YidD-expressing cells were subjected to isopycnic sucrose gradient centrifugation for separation of IMs and OMs (Fig. 3B). Analysis of the fractions by Western blotting revealed a similar distribution for His-YidD and the control IMP Lep, peaking in fractions 9 and 10 and 13 to 16, while TolC, a control outer membrane protein (OMP), as almost exclusively localized in fractions 8 to 11. Despite the observation that the more dense OM fractions seemed slightly contaminated with IMs (but not vice versa), the data clearly indicated that His-YidD is located in or at the IM. Of note, the same distributions of Lep and TolC were observed in membranes derived from cells harboring the empty plasmid pEH3, indicating that His-YidD expression did not interfere with the membrane separation (data not shown). To rule out that His-YidD forms aggregates that cofractionated with E. coli membranes, crude membranes were also subjected to sucrose floatation gradient analysis, showing that His-YidD, similar to Lep, floated with the IMs (data not shown).

To independently corroborate the IM localization of His-YidD, crude membranes were subjected to differential solubilization using the detergent sodium lauryl sarcosinate (Sarkosyl) that preferentially solubilizes IMs (6). The results shown in Fig. 3C indicate that His-YidD, like the control IMP Lep, was solubilized by Sarkosyl in contrast to the control OMP TolC, which was primarily found in the nonextracted pellet fraction, as expected. Only part of His-YidD was recovered after the extraction procedure, which is probably due to the inherent instability of this tagged protein, as discussed above. Together, the data argue that His-YidD is associated with the IM.

To exclude the possibility that the membrane localization of His-YidD was influenced by the His tag, we replaced the His tag by green fluorescent protein (GFP). The latter fusion protein allowed a more direct evaluation of the location by fluorescence microscopy (Fig. 4B). MC4100-A cells producing GFP-YidD clearly showed a halo-type fluorescence, indicating that YidD localized in the cell envelope and most likely at the IM. In contrast, expression of nonfused GFP resulted in a diffuse signal throughout the cells, consistent with its expected cytoplasmic localization.

As mentioned before, YidD does not contain any hydrophobic domains to account for its membrane targeting and insertion. Secondary structure prediction did, however, reveal the presence of two to three potential α-helical regions (Fig. 1C). The most N-terminal of these is relatively conserved and displays a predicted amphipathic character (Fig. 1D). To investigate whether this helix acts as a membrane targeting sequence for YidD, we fused it directly to GFP (Fig. 4A). Indeed, this construct appeared to localize at the cell envelope (Fig. 4B), suggesting that the N-terminal amphipathic helix is responsible for the membrane localization of YidD. The membrane association appeared to withstand extraction with 4 M urea, suggesting that it represents a direct interaction with lipids since this treatment would interfere with protein-protein interactions (data not shown).

Function of YidD in IMP biogenesis. To examine the function of YidD, a chromosomal knockout mutant was constructed by deleting almost the entire coding sequence accord-

FIG. 3. His-YidD is localized at the IM. (A) Subcellular fractionation of MC4100-A containing pEH3His-YidC. Cells were grown in LB medium and induced with 100 μM IPTG for 1 h and fractionated, and the fractions were analyzed by Western blotting using antisera against the His tag and the control IMP Lep. T, total cell sample; D, cellular debris (pellet from low-speed centrifugation); S, soluble fraction (supernatant of high-speed centrifugation); M, membrane fraction (pellet of high-speed centrifugation). Four times the amount of material of the total cell sample was loaded for fractions D, S, and M. (B) Membranes from MC4100-A expressing His-YidD were separated by sucrose gradient centrifugation. Fractions were analyzed by Western blotting using antisera against the His tag, the control IMP Lep, and the control OMP TolC. (C) Membranes from MC4100-A expressing His-YidD were treated with 0.5% Sarkosyl to solubilize the IMs and centrifuged to collect the OMs. Samples of the fractions, derived from equal amounts of cell material, were analyzed by SDS-PAGE and Western blotting using antisera against the His tag, the control IMP Lep, and the control OMP TolC. M, total membranes; IM, inner membrane (supernatant of Sarkosyl extraction); OM, outer membrane (pellet of Sarkosyl extraction).
To study a possible functional relationship with YidC, we studied the membrane integration of three YidC-dependent IMPs: the chimeric protein M13P2 that consists of phage M13 procoat extended at its C terminus with the periplasmic P2 domain of Lep, the CyoA subunit of the cytochrome o oxidase complex, and the F₁,Fₒ-ATPase complex. Membrane insertion of M13P2 is exclusively dependent on YidC and can be conveniently monitored by processing of its signal peptide by the lipoprotein-specific signal peptidase II (45). Using Western blot analysis under steady-state conditions, we monitored the processing of both pre-M13P2 and HA-tagged pre-CyoA expressed from expression plasmids introduced in the WT and ΔyidD strains (Fig. 5B and C). Importantly, the HA tag has been shown not to interfere with insertion and maturation of CyoA (45).

In WT cells, most pre-M13P2 was processed to mature form (89% ± 2.1%), whereas pre-M13P2 significantly accumulated in ΔyidD cells resulting in only 69% ± 3.9% mature M13P2 of the total M13P2 produced (Fig. 5B). Endogenous Lep was probed on the same blot to serve as a loading control. Pre-CyoA-HA was also less efficiently processed in the ΔyidD strain (26% ± 3.2%) than in the WT background (43% ± 2.6%) (Fig. 5C). As a control, signal peptide processing of the Sec-dependent but YidC-independent outer membrane protein A (OmpA) appeared unaffected, demonstrating that indirect inactivation of the Sec translocon or signal peptidase had not occurred under these conditions (Fig. 5C). In addition, OmpA served as a loading control in this experiment.

Insertion of the F₁,Fₒ subunit of the F₁,Fₒ-ATPase complex is exclusively dependent on YidC which results in a severely reduced level of F₁,Fₒ in YidC-depleted IMVs (47). To study the effect of YidD, the level of F₁,Fₒ was analyzed by Western blotting in IMVs derived from the ΔyidD strain and its isogenic parental strain (Fig. 5D). Compared to the control protein Lep, a clear reduction of F₁,Fₒ was apparent upon inactivation of YidD.

Together, the data demonstrate that YidC-dependent membrane insertion is significantly affected but not completely
abolished in the absence of YidD, suggesting a functional link between the two proteins.

YidD cross-linking to nascent FtsQ. Previous in vitro site-specific cross-linking experiments have shown interactions of nascent IMPs with the translocon components SecY, SecA, and YidC that depended on the length of the nascent chain (36). In this approach, membrane insertion intermediates are generated by the translation of truncated mRNAs that lack a stop codon, thus preventing the release of the polypeptide chain from the ribosome. Considering the localization of YidD at the IM and the apparent role of YidD in YidC-dependent membrane insertion, we hypothesized that YidD is adjacent to a nascent model IMP during membrane insertion. Therefore, we chose to analyze nascent chains of FtsQ with a length of 108 amino acids (108FtsQ). FtsQ is a type II IMP that spans the IM once and plays a critical role in cell division. The TM of the well-characterized 108FtsQ intermediate is located between residues 24 and 49, exposed outside the ribosome, and has been shown to interact with YidC (36, 55). Because YidD contains three conserved cysteine residues, we decided to use sulfhydryl-specific cross-linking upon introduction of single cysteine residues in 108FtsQ to probe a putative interaction.

The cysteines were placed at positions 15 (located in the cytoplasmic domain), 36 (in the TM), and 61 (in the periplasmic domain) (Fig. 6B), all expected to be exposed outside the ribosome exit tunnel that covers approximately 35 residues.

Nascent chains of the three single cysteine FtsQ constructs were prepared by translating truncated mRNA in the presence of [35S]methionine in a cell- and membrane-free E. coli lysate. Purified inner membrane vesicles (IMVs) from WT cells and cells that produce His-YidD were added to the translation reaction mixture to allow membrane targeting and insertion of the translation intermediates. Subsequently, the samples were incubated with the homo-bifunctional cysteine-specific reagent BMOE to induce cross-linking followed by carbonate extraction to enrich for membrane-integral proteins. Adducts of ~25 kDa were specifically detected upon cross-linking of 108FtsQ-Cys15 in His-YidD IMVs (Fig. 6A, lane 4). IP experiments using antibodies directed against the His tag confirmed that the detected adduct, indeed, contained His-YidD cross-linked to 108FtsQ-Cys15 (Fig. 6A, lanes 1 to 2; the adduct is indicated by a bracket). The doublet of adducts might be caused by the observed heterogeneity in nascent chain size. Alternatively, it might reflect the involvement of different cysteine residues in YidD in cross-linking to 108FtsQ-Cys15, which could result in distinct adducts of slightly different gel mobilities. In contrast, 108FtsQ-Cys36 and 108FtsQ-Cys61 did not generate any specific cross-linked adducts in His-YidD compared to WT IMVs (Fig. 6A, lanes 5 to 8). These data suggest that His-YidD is in close vicinity of the nascent chain of FtsQ, presumably near the cytoplasmic N-terminal part of the membrane-inserted nascent chain and possibly to assist YidC with the insertion of the growing polypeptide.

**DISCUSSION**

In our effort to understand the role of YidD in membrane protein insertion and assembly, we have analyzed the small yidD ORF that is located directly upstream of yidC. YidD belongs to a family of small (~80 amino acids) hypothetical proteins with a domain of unknown function 37 (DUF37) comprising three conserved cysteine residues (49). The yidD gene is present in all phyla of Bacteria. We showed that E. coli yidD is expressed giving rise to a membrane-associated protein. Although yidD is not essential, we obtained evidence that its gene product is functionally related to YidC: in the absence of YidD the insertion and processing of three YidC substrates are
affected, and YidD was found cross-linked to a nascent IMP that is also in vicinity of YidC (55).

The rpmH-mpa-yidD-yidC-trmE gene cluster belongs to the most conserved clusters in Eubacteria and even Archaea (51). Such a strong conservation of gene proximity and order could point to a coordinated expression and a related function, which may be essential. We propose that this cluster functions in the cotranslational insertion of membrane proteins via YidC, which is considered to be the most ancient protein insertase (33). Interestingly, in several cases there is a partial overlap in the genes, the most extreme example being the complete overlap of the rpmH and mpa genes in the genus Thermus (14). Although small deviations exist in the presence and order of genes in this cluster within various microorganisms, yidC is usually preceded by yidD, and it is also cotranscribed with yidC (this study). Interestingly, in the genome of Blochmannia pennsylvanica the yidD gene is fused to the 5′ end of the yidC gene. B. pennsylvanica is an obligate intracellular symbiotic bacterium of the ant Camponotus pennsylvanica (9). The B. pennsylvanica genome consists of only 792 kb that resulted from a massive reduction of genome size compared to its free-living ancestors. The presence of the yidDC gene fusion among the only 610 identified ORFs in this genome strongly suggests an evolutionary pressure to maintain a linked function of the two proteins.

Despite the absence of a putative transmembrane anchor, all our analyses, including cell fractionation and differential detergent solubilization, indicated that YidD localized to the E. coli IM. Most likely, the predicted amphipathic α-helix is responsible for membrane targeting as it is sufficient to target GFP to the membrane. In contrast to YidC, which accumulates responsible for membrane targeting as it is sufficient to target GFP to the membrane. In contrast to YidC, which accumulates individually, His tagged YidD fused to GFP showed a circumferential distribution at the cell periphery that is consistent with a dispersed localization in the cell envelope. It should be noted that this chimeric construct is probably present in amounts that exceed the endogenous YidD level. Interestingly, YidD from the obligate intracellular Gram-negative pathogen Chlamydia trachomatis has been shown to associate with lipid droplets when expressed in yeast (29, 37), suggesting that affinity for lipids may be a general feature of YidD orthologs.

What is the molecular environment of YidD in the membrane, and what is its function? His-tagged YidD was cross-linked to the cytosolic domain of the nascent chain of 108 residues of the IMP FtsQ, indicating a location of YidD at the cytosolic side of the IM. In fact, the molecular environment of the 108FtsQ insertion intermediate used is well characterized in cross-linking and structural studies (18, 36, 43, 55) and also includes YidC and parts of the Sec translocon. The combined data indicate that at this stage in translation and insertion of FtsQ, the TM is close to YidC and membrane lipids, whereas the flanking regions interact with SecY and YidD (36, 43) (Fig. 6B). Assuming that the FtsQ intermediate has adopted a stable homogeneous location, this would imply that YidD is also close to SecA, SecY, and perhaps YidC and the ribosomal nascent chain exit region. However, pulldown experiments using cross-linked and non-cross-linked cells and membranes have failed to indicate a physical connection between YidD and components of the ribosome and Sec-YidC translocon (data not shown). Although these negative data are difficult to interpret, they would indicate that any interaction of these components with YidD, if existing, is transient and/or of low affinity.

At present, we can only speculate about the function of YidD. The cross-linking of YidD to the N terminus of nascent 108FtsQ, concomitant with its proximity to YidC, SecA, and SecY, might suggest that YidD functions as an interaction partner, perhaps as a chaperone for the cytosolic part of nascent IMPs during membrane insertion. The basic YidD, positively charged at physiological pH, might also assist in tethering the ribosome to the membrane. It has been shown that the positively charged C-terminal domain of YidC contributes to ribosome binding (28), but this tail is much shorter than the corresponding C-terminal extension of the homologous mitochondrial Oxal protein that is both required and sufficient for ribosome binding during cotranslational membrane insertion in mitochondria (25, 40).

Currently, the role of YidD is being analyzed in more detail by studying proteome-wide effects of YidD deletion and by extensive in vitro cross-linking analyses.

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