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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Geng, Y. (2015). Membrane protein insertases of the YidC/Oxa1/Alb3 protein family [Groningen]: University of Groningen

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Chapter 1

Membrane protein insertases of the YidC/Oxa1/Alb3 protein family

Yanping Geng and Arnold J.M. Driessen
Membrane protein insertases of the YidC/Oxa1/Alb3 protein family

Abstract

Members of the YidC/Oxa1/Alb3 protein family are ubiquitous throughout the different domains of life. They play important roles in the membrane biogenesis of inner membrane proteins in bacteria, mitochondria and chloroplasts. In bacteria, most inner membrane proteins are integrated into the membrane via a co-translational mechanism. YidC can function as an independent membrane protein insertase, but it also cooperates with the SecYEG translocon whereby it assists in the folding and/or assembly of inner membrane proteins. Recent structural and functional studies provide a possible mechanism by which inner membrane proteins are integrated into the membrane by YidC. YidC possesses a conserved core domain of five transmembrane segments, which assemble into a hydrophilic groove at the cytoplasmic leaflet of the membrane. This groove is supposed to be the catalytic center of membrane insertion. Here, we summarize the common structural and functional aspects of the YidC/Oxa1/Alb3 protein family, including specialized functions of YidC paralogs.
1. Introduction

Membrane proteins are ubiquitous in all living organisms, making up about 20%~30% of the total synthesized proteins in cells (1). They perform a variety of functions including energy transduction and generation, signal transduction, ion and molecule transport, motility, metabolism and photosynthetic reactions. To execute their functions, newly synthesized membrane proteins need to be targeted to their membrane destinations where they can fold and/or assembly into a functional unit.

Integral membrane proteins span the membrane, mostly by α-helices that form individual hydrophobic transmembrane segments (TMSs) (1). Membrane proteins are inserted into the membrane via the conserved Sec translocase: SecYEG in the cytoplasmic membrane of bacteria and Sec61αβγ in the endoplasmatic reticulum (ER) membrane of eukaryotes (2, 3). This process occurs mostly via a co-translational mechanism (Fig. 1) (4). Herein, a ribosome nascent chain complex (RNC) is targeted to the membrane by signal recognition particle (SRP), which recognizes and binds to a TMS of the nascent inner membrane protein (IMP) once it emerges from the ribosomal exit tunnel (5). The SRP-bound RNC is then directed to the membrane receptor FtsY (or SRα/SRβ in eukaryotes), where GTP hydrolysis drives the transfer of the RNC to the SecYEG protein-conducting channel. Soon afterwards, the SRP/FtsY heterodimeric complex dissociates to release SRP for another round of membrane targeting (2, 6). In bacteria, not all of the IMPs employ the SecYEG translocon for membrane insertion. A small subset of the membrane proteins, especially small IMPs with very hydrophobic TMSs, require YidC for membrane biogenesis (7). Members of the evolutionarily conserved YidC/Oxa1/Alb3 protein family are universal in bacteria, euryarchaeota, and eukaryotes (8). They can function as independent insertases for the membrane insertion of bacterial and mitochondrial inner membrane proteins, and thylakoid membrane proteins of chloroplasts (6). Alternatively, they function as chaperones in facilitating protein folding and/or assembly during membrane insertion. One common feature of the YidC/Oxa1/Alb3 protein family is their involvement in the insertion and assembly of the multimeric energy transduction complexes such as the F$_{1}$F$_{0}$ATPase (9). Therefore, YidC-like proteins play a significant role in cell physiology.
Figure 1. Schematic representation of protein insertion pathways in bacteria. Bacterial inner membrane proteins (IMPs) are inserted into the lipid bilayer via a co-translational insertion pathway. (A) The majority of newly synthesized IMPs are targeted to the SecYEG translocon in a SRP/FtsY dependent manner where the IMPs concomitantly with polypeptide chain elongation at the ribosome, and insert into the lipid bilayer via SecYEG. YidC occupies the lateral gate of the SecYEG translocase, possibly facilitating the release of the hydrophobic TMSs from the SecYEG protein conducting channel into the lipid phase, and/or the proper folding and assembly of the IMPs. YidC can also function as independent insertase for IMPs either in conjunction with RNCs (A) or posttranslational (B). Membrane insertion of some IMPs is also dependent on the proton motive force. (C) During post-translational targeting, fully synthesized preproteins are kept in a translocation competent state by the molecular chaperone SecB and targeted to the motor protein SecA bound to the SecYEG translocon. SecA drives the translocation of preproteins across the membrane via multiple ATP hydrolysis cycles.

Recently, high-resolution crystal structures of a YidC homolog from *E. coli* and *Bacillus halodurans* have been obtained that provide insight into the mechanism of YidC-mediated insertion (10, 11). The conserved C-terminal region of YidC comprising five TMSs that are arranged into a hydrophilic groove which is sealed at the periplasmic side while open to both the cytoplasm and the membrane. This structural insight now for the first time provides a glimpse on how membrane proteins may slide into the membrane at the YidC protein to lipid interface. In the review, we will describe the distribution, common structural and functional features of the YidC/Oxa1/Alb3 protein family. Our current understanding of dynamic in-
teractions of YidC with other protein factors involved in membrane biogenesis will also be discussed.

2. The YidC/Oxa1/Alb3 family: Phylogenetic analysis

Members of the YidC/Oxa1/Alb3 protein family are distributed ubiquitously in all domains of life. As the phylogenetic tree reveals (Fig. 2), gene duplications have led to six sub-branches of the YidC/Oxa1/Alb3 protein family: YidC1 and YidC2 in bacteria, Oxa1 and Oxa2/Cox18 in mitochondria, and Alb3 and Alb4 in chloroplasts. All of the YidC homologs are evolved from a common ancestor. In bacteria, most Gram-negative bacteria, such as *E. coli*, contain only one YidC homolog (YidC), while two copies are found in many Gram-positive bacteria (YidC1 and YidC2). In eukaryotes, YidC homologs are found in the inner membrane of mitochondria (Oxa proteins) and the thylakoid membrane of chloroplasts (Alb3 proteins) with a number ranging from one to six. YidC homologs are absent from the plasma and ER membrane. The yeast *Saccharomyces cerevisiae* possesses only one copy of a Oxa protein, while fungi and metazoan have two copies. Six copies were identified in the plant *Arabidopsis thaliana* (8), but not all eukaryotes seem to possess YidC-like proteins, such as the mitochondrial protozoa *Giardia lamblia* and *Trichomonas vaginalis* (12). It is assumed that the rich diversity of the YidC homologs in eukaryotes arises from independent gene duplications or loss during evolution. In archaea, a single copy of a YidC homologue is found but only in some, and not all, euryarchaeota. All of the putative YidC homologs are predicted to possess four conserved TMSs with a rather low similarity to the bacterial YidC both in the sequence and the length (8, 12). Since there is no functional data on the identified archaeal YidC homologs, the exact significance of the sequence similarity is still unclear. Thus, it appears that YidC was lost in most archaea while it diverged in some euryarchaeota. In this respect, Archaea and Eukaryotes emerged from the same branch during evolution, prior to the formation of mitochondria and chloroplasts that evolved from a prokaryotic endosymbionts. The latter event likely introduced YidC into these eukaryotic organelles.

During evolution of the YidC/Oxa1/Alb3 protein family, independent gene duplications result in YidC paralogs with specialized functions in one organism or organelle. In *B. subtilis*, YidC1 (SpoIIIJ) is essential for sporulation and YidC2 (YqiG) is involved in competence de-
development. These specific functions are not exchangeable (13) in contrast to their function during vegetative growth. For some YidC-like proteins, a long hydrophilic stretch, proposed to be involved in membrane targeting, has been added to the C-terminus in the event of gene duplication. In mitochondria, a long C-terminal extension enables Oxa1 to support ribosome binding during co-translational insertion, whereas its paralog Oxa2/Cox18 lacks this extended region and its activity is restricted to post-translational insertion (14, 15). The Streptococcus mutants YidC2 contains a similar ribosome binding C-terminal tail, which was found to partially compensate for a SRP defect suggesting a conserved role in ribosome targeting (16). The C-terminus of Alb3 is specific for recruiting the chloroplast signal recognition particle (cpSRP43) and the light harvesting complex protein (LHCP) to the thylakoid membrane (17). In summary, independent gene duplications not only enriched the diversity of the YidC/Oxa1/Alb3 protein family but also broaden their substrate spectrum, whereas early in evolution, YidC diverged or even got lost in the archaeal kingdom of life.

Figure 2. Phylogenetic tree of the YidC/Oxa1/Alb3 protein family. The YidC-like proteins in bacteria, mitochondria and chloroplasts clearly fall into three clades including six sub-branches. The figure was adapted from Funes et al. (2011). The archaeal homologs are not included in this phylogenetic tree as these are too divergent.
3. Common functions of the YidC/Oxa1/Alb3 protein family

YidC homologs are functionally conserved in catalyzing the membrane insertion, assembly and/or folding of the inner membrane proteins of bacteria, mitochondria and the thylakoid membrane proteins in chloroplasts (18–22).

The YidC homolog of mitochondria, Oxa1, was first discovered to be involved into the membrane biogenesis of mitochondrial IMPs. Oxa1 was initially identified in yeast as an essential factor for the assembly of cytochrome c oxidase, as depletion of Oxa1 resulted in a respiratory deficiency phenotype related to an undetectable cytochrome oxidase complex (23). Later, it is found that Oxa1 tightly interacts with the nascent polypeptide chains of subunit 1 (Cox1), subunit 2 (Cox2) and subunit 3 (Cox3) of the cytochrome oxidase (COX) complex as well as the cytochrome b (Cytb) of cytochrome c reductase during co-translational insertion. This provides evidence that Oxa1 plays an important role in membrane protein biogenesis (24–27). Cox18 (termed Oxa2 in N. crassasa) represents another subgroup of the YidC homologs of mitochondria, and this protein was shown to be essential for the membrane biogenesis of the COX complex by promoting the translocation of the C-terminus of Cox2 across the inner membrane of mitochondria (28). Therefore, the Oxa1/Cox18 paralogs are characterized as independent IMP insertases in mitochondria, and this function in membrane insertion is one of the common features of the YidC/Oxa1/Alb3 protein family (29, 30).

In 2000, Scotti and coworkers found that the E. coli YidC could be co-purified with the SecYEG translocase and cross-linked to the signal anchor sequence of a nascent membrane protein FtsQ, providing a hint for the involvement of YidC in the membrane insertion mechanism (31). Meanwhile, it was reported that the E. coli YidC is essential for viability and that it plays a critical role in the membrane insertion of the Sec-independent substrate Pf3, which is a coat protein of a phage (29). These two findings point out a novel membrane insertion pathway in bacteria mediated by YidC either independent or in conjunction with the Sec translocon. By means of an in vitro approach, YidC was shown to insert membrane proteins (32, 33). In the assay, the membrane insertion of a subset of IMPs was analyzed using proteoliposomes containing the purified YidC protein. The first endogenous E. coli substrate found was F, subunit of the F, ATPase complex. Further in vivo and in vitro approaches now define
Table 1 Identified substrates for the YidC/Oxa1/Alb3 protein family. Substrate specific functions of YidC are depicted in the bracket.

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a: Cox18 is termed Oxa2 in *Neurospora crassa* (15); b: the homologs of Alb3 in *Chlamydomonas reinhardtii* (63); c: carrier proteins (e.g. mitochondrial ATP/ADP carrier protein Aac2).
a small subset of the YidC-exclusive substrates (table 1). These are rather small proteins with very hydrophobic TMS(s) with negatively charged N- or C-termini or loops at the periplasmic face of the membrane (34).

In addition to the insertion activity, YidC homologs in bacteria, mitochondria and chloroplasts play crucial roles in the assembly of membrane protein complexes (14, 32, 35, 36). Depletion of YidC induced a dramatic reduction of the ability of cells to generate a proton motive force (PMF), which is due to defects in the assembly of the F₁F₀ATPase complex and cytochrome o oxidase (32). Proteomic studies using isotopic labeling showed that depletion of YidC induced an up-regulation of chaperone proteins, which are involved in processing misfolded and unassembled proteins (37). The amounts of membrane subunits of the energy-transducing protein complexes, such as CyoA and CyoB of cytochrome o oxidase, CydA of cytochrome d terminal oxidase, NuoA and NuoL of the NADH dehydrogenase, were decreased upon the depletion of YidC (37), suggesting a role in their assembly. By using a two-dimensional Blue Native/SDS-PAGE methodology, a comprehensive analysis of the YidC-depleted inner membrane proteome of E. coli was carried out (38). It revealed that YidC is involved in the assembly of respiratory chain complexes, including the F₁F₀ATPase, the cytochrome bo3 oxidase and the NADH dehydrogenase. The steady state membrane levels of these complexes are substantially decreased in the absence of YidC, in agreement with previous data (37, 38). In B. subtilis, the two YidC homologs (SpoIIIJ/YqiG) were found to copurify with the ATPase complex. Although a double depletion of SpoIIIJ/YqiG slightly affected the membrane insertion of F₀ subunits, the activity of the ATPase complex and the level of membrane associated F₁-sector were dramatically reduced, suggesting that SpoIIIJ/YqiG function as chaperones in the assembly of the ATPase complex possibly by docking the cytosolic F₁-sector onto the membrane integrated F₀-sector (35). This assembly activity seems a generic feature of the YidC/Oxa1/Alb3 protein family, as deletion mutants lacking Oxa1 or Alb4 also showed defects in the assembly of the ATPase complex (36, 39). The assembly of Atp9 (F₀c) with the F₁-sector was not influenced by Oxa1 depletion in yeast, but further association of Atp6 (F₀a) with the Atp9 (F₀c)-F₁ subcomplex was blocked (39). An alb4 null mutant of A. thaliana showed reduced activity of the CF₁F₀-ATP synthase due to an assembly deficiency. Alb4 depletion reduced the levels of subunits of the CF₁F₀-ATP synthase, and Alb4
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was found to interact with the CF$_{1}$β and CF$_{3}$II subunits. *Chlamydomonas reinhardtii* Alb3 paralogs (Alb3.1 and Alb3.2) also play crucial roles in the assembly of photosystems PSI and PSII (40). The assembly activity of the YidC/Oxa1/Alb3 protein family is likely related to insertion, as during insertion the interaction of the YidC-like proteins with the membrane subunits of the oligomeric complex may stabilize the assembly competence of folding intermediates. On the other hand, the bacterial YidC has been shown to be directly involved in the folding of polytopic membrane proteins (41–43). YidC is shown to be essential for the folding of the integral membrane subunit MalF of the maltose transport complex MalFGK2, and of the lactose permease (LacY). Both IMPs insert into the membrane via the Sec translocon.

Members of the YidC/Oxa1/Alb3 protein family are to some extent functionally exchangeable. Complementation studies have shown that both Alb3 and Oxa1 are able to substitute for the *E. coli* YidC in membrane protein insertion and vice versa (44–46). Alb3 of *A. thaliana* is able to functionally replace the *E. coli* YidC in both YidC- and the SecYEG/YidC-mediated insertion pathways, while the yeast Oxa1 could only compensate for the YidC insertase activity independent of the Sec translocon (44, 45). In addition, both Oxa1 and Cox18 in yeast can be partially replaced by the *E. coli* YidC, but in the case of Oxa1, it was necessary to fuse YidC to the long C-terminal ribosome binding domain of Oxa1 (46).

To summarize, the YidC homologs in bacteria, mitochondria and chloroplasts are functionally involved in IMP membrane insertion, folding and/or assembly, and these functions are largely exchangeable within the protein family across the domains of life.

4. **Bacterial YidC**

4.1. **Physiological function of YidC**

The *E. coli* YidC is the best-studied member of the bacterial YidC homologs, which is essential for cell viability and membrane biogenesis (29). In the absence of YidC, cell growth cessation has been observed under both aerobic and anaerobic conditions, which appears primarily due to a deficient membrane biogenesis of the respiratory chain complexes (64, 65) causing a loss of the proton motive force. Also, stress responses are triggered upon YidC depletion. A typical stress response to YidC depletion is the substantial expression of the phage shock protein (PspA). PspA binds to the lipid surface to maintain the proton motive force (PMF)
by preventing the proton leakage (66). Its high level expression suggests massive damages to the membrane and a dissipation of the PMF. Also an up-regulation of the Cpx/σE pathway has been detected upon YidC depletion, which is possibly caused by the accumulation of non-assembled inner membrane proteins (67). In addition, YidC has been proposed to be involved in the quality control of membrane proteins. FtsH functions as a protease in degrading mis-assembled membrane proteins. YidC interacts with a membrane complex including FtsH and its modulator HflK/C (68). Comprehensive proteome studies of YidC-depleted inner membranes show increased levels of FtsH, HflC and HflK, as well as a number of other chaperone proteins, like DnaK, PpiD, OppA (37, 38). Microarray data allow to identify the up-regulation of the quality control systems under those conditions, including the clpB, groES and groEL genes (69). YidC is of importance for cell morphology as YidC depletion impairs cell division leading to elongated cells (59, 69). The latter is likely related to a folding defect of a number of penicillin binding proteins that fulfill a critical role in peptidoglycan biosynthesis.

In contrast to Gram-negative bacteria, Gram-positive bacteria contain two YidC homologs (YidC1 and YidC2) (70). In *B. subtilis*, they are SpoIIIJ and YqjG, one of which is sufficient for cell viability but the concomitant deletion of the two paralogs is lethal to the cells (71, 72). Double deletion of the two *B. subtilis* paralogs induced a strong stress response which is not observed for the single deletions (13). This concerned the up-regulation of the LiaH, HtpX, YdjF and YokG proteins. Elevated levels were observed for LiaH, a cell-wall stress response protein that has sequence similarity with the *E. coli* PspA. SpoIIIJ is expressed constitutively during vegetative growth, while YqjG is induced when SpoIIIJ is absent or dysfunctional (73, 74). The level of YqjG within the cell is regulated by a SpoIIIJ activity sensor MifM, which is a SpoIIIJ-dependent single-spanning membrane protein (74, 75). Under SpoIIIJ-limiting conditions, translational stalling of MifM releases the Shine-Dalgarno sequence of the downstream *yqjG* gene, and thus induces an up-regulated expression of *yqjG*. A recent study indicates that the translational arrest of MifM could also be released by YqjG, accomplishing a feedback inhibition to maintain the cellular levels of YqjG (75).

SpoIIIJ (YidC1) and YqjG (YidC2) possess the conserved functions of the YidC/Oxa1/Alb3 protein family in membrane biogenesis (35, 74). They are able to catalyze the insertion and assembly of specific subunits of the $F_1F_0$ATPase complexes of both *E. coli* and *B. subtilis*, and
compensate for the growth defect of an *E. coli* YidC depletion strain (35). However, SpoIIIJ and YqjG have specific functions during *B. subtilis* development. SpoIIIJ is specific for sporulation, and YqjG enhances competence development (35, 76). During sporulation, SpoIIIJ is essential for the activation of the late forespore-specific sigma factor σ\(G\). Otherwise, sporulation is arrested at stage III after engulfment completion. Activation of σ\(G\) also requires expression of the SpoIIIA complex composed of the membrane subunits of the SpoIIIAA-SpoIIIAH, and a functional SpoIIIAH-SpoIIQ channel (77). Depletion of SpoIIIJ results in a non-functional SpoIIIAE (78). In addition, SpoIIIJ has been suggested to be involved in the membrane folding or assembly of the SpoIIIAH-SpoIIQ channel. An up-regulation of the single-spanning membrane protein SpoIIIAH has been observed in a *spoIIIJ*-null mutant, suggesting that SpoIIIJ might play a role in folding rather than insertion of SpoIIIAH. However, this hypothesis needs to be validated in experiments that demonstrate that SpoIIIAH is a specific substrate for SpoIIIJ (YidC1). To identify the determinant for the specific function of SpoIIIJ in sporulation, a series of SpoIIIJ-YqjG and YqjG-SpoIIIJ chimeras have been constructed and subject to sporulation investigation (79). TMS2 with its flanking hydrophilic regions of SpoIIIJ plays a crucial role in sporulation, and in cooperation with TMS1 and TMS3 support efficient sporulation. Residue C134 in TMS2 of SpoIIIJ is important for efficient sporulation by stabilizing a dimeric form of SpoIIIJ (80). However, the mechanism by which SpoIIIJ fulfills this specific function in sporulation is still unknown.

### 4.2. Structural and functional domains of YidC

The membrane topology of the bacterial YidC homologs are ubiquitously conserved. These proteins contain a conserved core comprising five TMSs connected by hydrophilic loops. In Gram-positive bacteria, the YidC homologs (YidC1/YidC2) possess a putative lipoprotein signal peptide at the N-terminus, which is removed by the signal peptidase II following membrane insertion (70). While YidC in Gram-negative bacteria have an additional N-terminal TMS that precedes the large periplasmic domain (8, 18).

#### 4.2.1. Structure of YidC

From an initial cryo-electron microscopy (cryo-EM) study, YidC was proposed to oligomerize into a homodimer, forming an insertion channel at the dimeric interface (81). By using the high sensitive single molecule techniques of the fluorescence cross correlation spectroscopy
(FCCS), it was demonstrated that a monomeric YidC in the membrane or in detergent solution is sufficient for ribosome binding defining a minimal functional unit (82). This was verified by a recent cryo-EM structure of a YidC-ribosome complex at 8.6Å that revealed a YidC monomer located at the ribosomal exit tunnel carrying a nascent polypeptide chain (83). A breakthrough in the field was the crystal structure of YidC. Kumazaki and coworkers reported the high-resolution structures of a YidC homolog from *Bacillus halodurans* (YidC2) and the *E. coli* YidC (Fig. 3) (10, 11). Both structures were elucidated in lipid cubic phases. It suggests that the bacterial YidC exists as a monomer in the membrane and facilitates the insertion of membrane proteins by a channel-independent mechanism.

In the crystal structures, the global arrangement of the conserved five TMSs of YidC are in high agreement (Fig. 3). They are tightly packed in the outer leaflet of the membrane and loosely interact with each other in the inner leaflet of the membrane, folding into a hydrophilic groove that is proposed to accommodate the polar regions of substrates. A conserved positively charged arginine resides (Arg72 in *B. halodurans* YidC2 and Arg366 in *E. coli* YidC) in the middle of the cavity, which is of functional importance (10, 84). Except for the replacement with lysine, other substitutions for the conserved arginine in *B. subtilis* SpoIIIJ (Arg73) compromised its functionality in complementing the growth defects of a *spoIIIJ* deletion

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**Figure 3** Structure of the *E. coli* YidC. (A) Ribbon representation of the *E. coli* YidC structure (PDB ID: 3WVF). Viewed transversely through the membrane. (B) Superimposition of the crystal structures of YidC from *E. coli* (colored) and *B. halodurans* (PDB ID: 3WO6, grey). In comparison with that of *B. halodurans* YidC, the C1 hairpin of *E. coli* YidC is rotated by ~35° with respect to the TMSs.
strain and membrane insertion of MifM (10). In contrast, the conserved arginine (Arg366) in the \textit{E. coli} YidC is more tolerant to mutagenesis. Under normal growth conditions, mutation of Arg366 with a neutral or positively charged residue has no effect on the YidC activity, and only a negatively charged residue at that position compromises the YidC functionality in growth complementation of an \textit{E. coli} YidC depletion strain and in membrane insertion of Pf3-23Lep. Similar observations were made for the \textit{A. thaliana} Alb3 (85). The conserved arginine in YidC-like proteins appears functionally important under certain conditions only, such as at a low temperature (20 °C) for \textit{E. coli} YidC. In addition, mutation of the corresponding arginine in yeast Oxa1 (R140A) resulted in a cold-sensitive growth phenotype (86). On the other hand, the positive charge of arginine is proposed to attract the negative region of substrates, because elimination of the negatively charged residues in the N-terminal tail of MifM and the Pf3 coat protein dramatically impaired their membrane insertion (10). It is proposed that the electrostatic interaction between the positively charged arginine in the hydrophilic groove of YidC and the negatively charged regions of substrates play a crucial role in membrane insertion, but this function may relate to a subset of substrates only. In agreement with the crystallographic analysis, a recent \textit{in vivo} study suggests that SpoIIIJ provides a hydrophilic environment in the membrane that is primarily contributed by hydrophilic residues of the TMSs at the cytoplasmic side of the membrane (87). Replacement of these hydrophilic residues with non-polar ones compromised the water accessibility and functionality of SpoIIIJ, implying a functional importance of the aqueous environment for YidC-like proteins. The study also pinpoints the functional requirement for a positively charged residue in the hydrophilic groove, but an accurate positioning seems not necessary because relocation of Arg72 inside the cavity of SpoIIIJ has no effect on its activity.

Based on these findings, a model has been proposed on how YidC inserts monotypic membrane proteins into the membrane. Herein, membrane insertion is initiated at the positively charged cytosolic loop 1 in between TMS2 and TMS3 of \textit{E. coli} YidC (corresponding to TMS1 and TMS2 in \textit{B. halodurans} YidC2). Further incorporation of the substrates into YidC is attracted by the electrostatic interaction between the negatively charged residues of the substrates and the conserved arginine in the hydrophilic cavity of YidC (10, 84, 85). Final ‘sliding’ and membrane insertion of the TMSs of substrate are driven by the hydrophobicity of the
inserting TMS as well as the proton motive force. Although the model may describe how a single TMS containing proteins like Pf3 insert, it does not explain how bitopic membrane proteins like F$_0$c insert. Also, since YidC is closed at the periplasmic side, it is unclear how the hydrophobic regions of substrates are translocated across the sealed part. Wickles and coworkers proposed that YidC can reduce the thickness of the lipid bilayer to facilitate the insertion of hydrophobic TMSs into the lipid bilayer thereby reducing the energetic costs of membrane insertion (88). Importantly, in the model, YidC provides an aqueous microenvironment in the lipid phase to facilitate the translocation of the hydrophilic regions of substrate. However, the model does not yet include other factors that might contribute to membrane insertion, such as the interaction of YidC with the translating ribosomes.

4.2.2. Functional domains of YidC

X-ray crystallographic analysis has revealed that the large periplasmic domain (P1) of the *E. coli* YidC is packed into a β-supersandwich fold with a large cleft residing on one surface. The cleft is oriented away from the membrane and possibly accommodates the periplasmic molecules or proteins rather than substrates (84, 89). An amphipathic α-helical linker that is parallel to the membrane plane orients the P1 domain to the membrane, and this architecture is stabilized by conserved interaction between the P1 domain and the P2 domain (the loop in between the TMS3 and TMS4 of YidC) (84, 89, 90). Notably, the P1 domain is not essential for YidC activity, as more than 90% of the region can be deleted without influencing the YidC functionality in cell viability and membrane insertion (91). A portion (the region 215-265) of the P1 loop, especially residue Lys249, could be cross-linked to SecG and the SecDF-YajC complex (92, 93). Therefore, the P1 domain is possibly involved in stabilizing the interaction between YidC and the Sec translocon.

The five core TMSs of YidC are arranged into a hydrophilic groove, which is open to both the cytoplasmic side and the membrane interior via the gap between TMS3 and TMS5. The cavity is assumed to attract and accommodate the hydrophilic region of substrates during insertion (84). Mutagenesis studies have elucidated that TMS2, -3 and -6 are crucial for *E. coli* YidC functionality, while TMS4 and TMS5 are highly tolerant to amino acid mutations (91). Two mutations, i.e. C423R and P431L in TMS3 resulted in a cold sensitive phenotype (94), and T362A in TMS2 and Y517A in TMS6 cause a lethal phenotype at 37°C (88). On
the other hand, cross-linking experiments have mapped a number of substrates-contacting sites on TMS3 of the *E. coli* YidC, including cross-links with the Pf3 coat protein, F₀c, and the Sec-dependent substrates LepB and FtsQ (95–97). These interaction sites are primarily mapped on the exterior surface of TMS3, TMS4 and TMS5, and are in close proximity to the opening of the hydrophilic groove.

The cytosolic loop 1 (C1) in between TMS2 and TMS3 of the *E. coli* YidC (TMS1 and TMS2 of *B. halodurans* YidC2) protrudes into the cytoplasm and folds into a hairpin-like structure (CH1 and CH2), which is parallel to the membrane plane (84). Deletions within the C1 domain of the *B. halodurans* YidC2 homolog SpoIIIJ severely impair cell growth and MifM insertion, implying a crucial role of the region in YidC activity. The C1 domain of the *E. coli* YidC is more flexible compared to that of the *B. halodurans* YidC (Fig. 3). It is suggested that the whole C1 region (371-416) is dispensable for YidC activity, while in another study deletion of the domain inactivated YidC for cell viability and membrane insertion of Pf3 and Procoat-Lep fusion proteins (85, 91). Recently, by the use of short deletions, region 374-383 of C1 domain of YidC is shown to be essential for cell viability (98), indicating a functionally important role of the hairpin like structure. The cytosolic loop 2 (C2) and the C-terminus of YidC are structurally disordered in the crystal structure. Both of the regions have been suggested to be involved in ribosome binding (88). Neither the C2 domain nor the C-tail is essential for YidC insertion activity (82, 91), whereas double deletions within the two regions resulted in a completely inactive YidC in membrane insertion for YidC-only substrates of F₀c and MscL(98). The latter suggests that double deletions possibly induce a conformational alteration that inactivates YidC in membrane insertion.

**4.3. Targeting of YidC-only substrates and ribosomal binding**

Co-translational membrane insertion is initiated by ribosome binding at the cytosolic face of the YidC protein (99). In YidC-mediated insertion pathway, nascent IMPs may be targeted via SRP and its membrane receptor FtsY, as was shown for MscL (100). Alternatively, IMPs can be directly targeted to the membrane via an electrostatic mechanism by the contact between the substrates and the lipids, like the M13 procoat protein (101), or interaction between the substrates and YidC as for instance the F₀c protein (102).

YidC may directly accept the nascent polypeptide chains by binding to the ribosome. In mito-
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Chondria, Oxa1 binds to the ribosome during co-translational insertion (14, 103). Cross-linking study revealed a contact of Oxa1 with Mrp20 and Mrp40, homologs of bacterial ribosomal proteins L23 and L24 that are located close to the ribosomal polypeptide exit site (103, 104). Interestingly, the physically interact of Oxa1 with ribosome is specifically attributed to its long and positively charged C-terminal extension (86 residues; +14 charge), which is absent in the paralog Cox18 (14). In S. mutant, YidC2 has a longer C-terminus (61 residues, +14 charge) compared to that of YidC1 (33 residues, +9 charge). However, both YidC1 and YidC2 have been demonstrated to interact with the E. coli ribosome via their C-terminal domains (105), but only the C-terminal extension of YidC2 can functionally complement the binding of Oxa1 to mitochondrial ribosome (70, 105). Furthermore, YidC2 can partially compensate for SRP depletion to support cell growth, possibly due to the targeting role of the extended C-terminus (16). Also the extended C-tail of YidC from Gram-negative bacterium Rhodopirellula baltica allows a tight binding of ribosome. A chimeric E. coli YidC protein appended with the C-tail of R. baltica YidC (YidC-Rb) shows a strong ribosome binding primarily at the ribosomal protein L29 (83). YidC-Rb also exhibits a reduced dependence on SRP for the membrane insertion of MscL, implying an overlapping function of the extended C-terminus of R. baltica with SRP/FtsY. Thus, a general feature of the longer C-terminus of YidC is to support ribosome binding and targeting the nascent IMPs during co-translational membrane insertion.

The E. coli YidC has a shorter C-terminal tail (13 residues; +4 charge) compared to its homologs. It is found that the C-terminus of YidC interacts with targeting components (SRP, SRP).

Figure 4. Cryo-EM structure of a YidC-RNC complex. Close up view of the contact area between the C1 and ribosomal H59 (A), the C2 and ribosomal protein L23 (B). TMSs of YidC are indicated in arabic numerals. The residues that are crucial for YidC activity are indicated by magenta spheres. The figure was adapted from Wickles et al. (2014).
FtsY), SecD, SecF and ribosomal subunits (81, 93, 106), but the binding with ribosome is questioned as it was studied under non-physiological conditions, where the protein was present in detergent solution. To rule out unspecific interaction, the binding of ribosomes to the membrane embedded YidC was studied, showing that monomeric YidC specifically binds to programmed ribosomes only, rather than empty ribosomes (82). In addition, detergent solubilized YidC binds empty ribosomes, but this interaction is likely not native. The positively charged C-terminus of YidC plays an critical role in efficient ribosome binding, but it is not the sole determinant. Even in the absence of the C-terminal tail, YidC retains a residual activity in ribosome binding. Therefore, other docking sites on YidC, like the two positively charged cytosolic domains are postulated to be involved in ribosome binding. Recently, a YidC structure model is constructed via an intramolecular co-variation analysis and fitted into the cryo-EM structure of the YidC:RNC-F$_{0}$c complex. This showed that the C1 and C2 loops of YidC associate with ribosomal RNA helix H59 and L23 protein (both are located at the ribosomal exit tunnel), respectively (88) (Fig. 4). The direct contact sites involved in ribosome binding are restricted to the residues Y370 and Y377 in the C1 domain, and D488 in the C2 domain (Fig. 4). Mutation of these residues severely compromised cell viability, suggesting that they are essential for YidC activity. To determine the regions of YidC that are responsible for ribosome binding, sequential deletions within the cytosolic loops of YidC were performed (98). The C2 loop and the C-terminus contribute to ribosome binding, and deletion of both not only interfered with ribosome binding but also inactivated the insertion of YidC-only substrates like F$_{0}$c and MscL.

4.4. Interaction of YidC with the Sec translocon

4.4.1. The SecYEG-SecDF-YajC-YidC holotranslocon complex

In bacteria, the majority of proteins are inserted into the membrane via the Sec translocon, which comprises a core heterotrimeric protein-conducting channel (composed of SecY, SecE and SecG) and the accessory proteins: the cytosolic motor protein SecA ATPase, the sub-complex SecDF-YajC and YidC (2, 107, 108). Detailed insight into the functioning of the SecYEG protein conducting channel were obtained from advanced biochemical studies, high-resolution crystal structures, and cryo-EM structures of SecYEG in complex with RNCs (109–117). The SecY channel has an hourglass-shaped structure formed by the two halves of TMSs1-5.
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and 6-10 of SecY that are connected by a loop between TMS5 and TMS6. SecE embraces the clamshell structure at the back of SecY to stabilize the two halves. SecG peripherally associates with the SecY channel, and it is non-essential for cell viability and translocation (118). At the center of the Sec channel is a constriction formed by a “pore ring” consisting of six hydrophobic residues, which is proposed to prevent permeation of ions or other small molecules during translocation (117). A small plug helix, formed by TMS2a of SecY that folds back into the funnel-like cavity, seals “pore ring” at the periplasmic side. Movement of the plug to the side opens the SecY channel to allow for translocation of preproteins (112). SecA is involved in translocation of preproteins and large hydrophilic domains in a stepwise manner by using multiple cycle of ATP binding and hydrolysis (2, 119). It is suggested that SecA binding triggers the displacement of the plug while it still occludes the SecY channel (110).

SecD, SecF and YajC are encoded in one operon, forming into a trimeric membrane complex that are in close contact with the SecYEG translocation channel (120, 121). SecDF has been proposed to function in later steps of translocation by releasing preproteins from the SecY channel into the periplasm (122) or prevent sliding back of preproteins inside the SecY channel to cytosol (120). Structural and biochemical studies suggest that SecDF contains 12 TMSs and two large periplasmic domains, and it is essential for the PMF-dependent translocation of preproteins (123). SecDFYajC is not essential for cell viability or protein translocation, but depletion or mutation of SecDF causes a cold-sensitivity phenotype for cells and severely impairs protein export (124). The role of SecDF in membrane protein insertion is unclear.

IMP5s are suggested to be inserted into the membrane via the lateral gate of the SecY channel formed by TMS2b, 3, 7 and 8 of SecY (117). Opening of the lateral gate is essential for partitioning of TMSs of IMP5s into the membrane, because crosslinking TMS7 and 2b that form the rim of the lateral gate blocks protein translocation (115). X-ray and cryo-EM studies have observed that signal sequences or TMSs of IMP5s intercalate between TMS2b and 7 of SecY and thus are proposed to leave the translocon via the lateral gate (113, 114, 117, 125, 126). Binding of ribosome or SecA and insertion of nascent chains are suggested to induce a “pre-opened” state of the lateral gate, which primes the Sec translocon for protein translocation (110, 113, 127–129). In the initial translocation stage, nascent polypeptide chains are supposed to insert as a loop in the SecY channel, with the signal anchor outside the lateral gate (113). One of
the two TMSs of a polypeptide nascent chain has been observed outside the lateral gate of a RNC-bound Sec translocon during translocation (126). These structural information of a ribosome-Sec complex exposing nascent chains provide insights into the mechanism of the SecYEG-mediated membrane insertion. However, understanding of the coordination between the SecYEG and other partners, such as ribosome, SecDF-YajC and YidC, will reveal detailed information on protein translocation.

YidC has been suggested to interact with the SecYEG-SecDFYajC complex to form a holoenzyme (31, 130). YidC is much more abundant compared to the Sec components, which would be in line with a dual function (131). In this supercomplex, YidC is proposed to bind to SecYEG via SecDF (121), and facilitates the exit of the hydrophobic TMSs of IMPs from the Sec channel into membrane. Cross-linking studies have shown that the hydrophobic regions of FtsQ and LepB sequentially interact with SecY and YidC (132, 133). YidC can be dynamically cross-linked to the lateral gate of SecY independent of SecDF, and the conserved residues G355 and M471 of YidC are possibly directly involved in the interaction of YidC with SecY (134). The YidC/SecY contacting sites at the lateral gate of SecY can be altered by ribosome or RNC binding but is not influenced by SecA binding (93).

In cooperation with SecYEG, YidC can act as a chaperone in providing folding sites for the multi-spanning substrates, such as MalK (8TMSs) and LacY (12TMSs) (42, 43, 135), and in providing assembly sites for the oligomeric complexes, like the F_{0}F_{1} ATPase and MalFGK (35, 43). Both YidC and SecYEG are required for the membrane insertion of F_{0}a and F_{0}b subunits of the ATPase (35, 56). As for CyoA of the cytochrome c oxidase, membrane insertion of the N-terminal signal sequence and TMS1 is YidC dependent, while membrane topogenesis of the C-terminal domain consisting of TMS2 and a large periplasmic loop requires YidC, SecYEG and SecA (52, 54). Translocation of the periplasmic loop1 and loop2 of TatC are also YidC-Sec-dependent (58). These data suggest that YidC and SecYEG cooperate closely in membrane biogenesis.

In a recent study YidC was copurified with SecYEG and SecDF as a holotranslocon complex (HTL) comprising a single copy of each component (136). The HTL supercomplex is active in both post- and co-translational pathways. Due to the presence of YidC, SecYEG is more effective in CyoA translocation compared to the SecYEG complex alone. However,
a complication in the study is that the co-purification was performed with histidine tags to YidC, SecD and SecE during co-expression, and also relies on his-tag purification as well as chemical crosslinking to retain the proteins in a complex. Therefore, it remains unclear if the holotranslocon exists as a stable complex or whether it is formed only transiently, and thus is not stable to detergent solubilization.

4.4.2. Substrate determinants for YidC and SecYEG

The characteristics of substrates that determine the insertion pathways have been extensively studied. It is proposed that unbalanced charge distribution around TMSs of IMPs is important for YidC dependence (137). Balancing the charge distribution around TMSs of CrcB (3 TMSs) allowed it to be inserted independent of YidC, while correcting the balanced charged distribution around TMSs of YaiZ (2 TMSs) resulted in a YidC-dependent protein. However, there must be other features of substrate that determine the insertion pathway, as many identified YidC substrates do not possess unbalanced TMSs. It is suggested that moderate hydrophobicity of TMS and charge composition of translocated regions function as determinants for insertion mechanism (138, 139). A correlation was found between the hydrophobicity of TMS of IMPs and the YidC requirement. Increasing the hydrophobicity of the YidC-only substrate Pf3 coat protein (1TMS) by replacing two alanine residues in TMS with threonine residues resulted in a YidC-independent insertion (138). By using a single-spanning model protein Pf3-Lep, it was observed that by decreasing the hydrophobicity of TMS, unassisted membrane insertion became YidC-dependent. Further mutational studies with this model protein proposed that charged residues in the periplasmic loop can be YidC determinants, while positive charge in the translocated region or TMS can act as a Sec determinant. The latter was confirmed by mutagenesis studies with the endogenous *E. coli* proteins F_{0a}, F_{0b} and TatC (139).

Below a certain threshold, the degree for Sec dependence of the YidC-only substrate MscL is correlated with the number of negatively charged residues in the periplasmic loop. Introducing 1, 2 or 3 negatively charged residues into the periplasmic domain of MscL gradually enhanced the Sec dependence, while extending the periplasmic loop with 3 positively charged arginine residues or 5 negatively charged glutamic acid residues inhibited membrane insertion (50). Latter studies also point out that charge composition and polarity of the periplas-
mic loops of IMPs are important determinants for insertion pathways (140). Increasing the number of charges or polarity of the translocated loop in M13-Lep procoat protein makes it require both YidC and SecYEG for membrane insertion, and it is the same case when adding charged residues to TMSs. In addition, increasing the hydrophobicity of TMSs allows M13-Lep to be inserted spontaneously, which strengthens the proposal that the hydrophobicity of TMSs can act as a selectivity determinant for YidC or the Sec insertion pathways (138, 139). Since there are only a limited number of substrates known for YidC, the elucidation of the features that direct these proteins into a YidC exclusive or a YidC-Sec pathway will be a difficult task.

In the Sec-YidC insertion pathway, YidC possibly assists in membrane folding or assembly of IMPs via electrostatic interaction between TMSs of YidC and inserting proteins. NuoK (3TMSs) is a polytopic membrane protein with two negatively charged glutamic acid residues in TMS2 and TMS3. NuoK requires both YidC and SecYEG for membrane insertion (57). Substitution of the two negatively charged residues for positively charged lysine residues rendered NuoK a SecYEG-only protein. The crystal structure reveals that YidC has a positively charged groove in the inner leaflet of membrane that is important for interacting with the polar regions of substrates, specifically for negatively charged translocated regions of substrates (10, 11). YidC possibly interacts with NuoK via electrostatic interactions between the positively charged arginine residues (Arg366) in the hydrophilic groove of YidC and the two negatively charged residues in NuoK(E36 and E72), and thus provides a transient amphipathic docking site for the TMSs of NuoK prior to folding and insertion into the membrane. Membrane insertion of CyoA (3TMSs) is also YidC/SecYEG dependent (53, 54, 141). CyoA does not contain any negatively charged residues in the TMSs, but the introduction of positively charged residues into the N-terminal hairpin domain rendered CyoA a SecYEG-only substrate (141), supporting the hypothesis that YidC functions in the lateral transfer of TMSs of inserting proteins into membrane via electrostatic interaction. However, the precise mechanism by which YidC facilitates lateral partitioning of TMSs remains largely enigmatic and requires further studies.

4.4.3. YidD, a new component in membrane insertion

In the E. coli genome, the yidC gene is located in a rather conserved five-gene cluster in the
order: rpmH, rnpA, yidD, yidC and trmE. This operon encodes proteins that are involved in protein synthesis and membrane biogenesis (142). RpmH encodes for the ribosomal protein L34 (5.4 kDa) that is in close vicinity of the ribosomal L23 (143), which provides the docking site for SRP, SecA, chaperone trigger factor and YidC at the ribosomal exit tunnel (88, 142). RnpA codes for the ribonuclease P (13.8 kDa) involved in RNA modification (144). TrmE encodes a GTPase subunit (49 kDa). The yidD gene is located in the center of the operon, and there is only 2 base pair gap with respect to the yidC gene. It can be co-transcribed with yidC suggesting a link between the two genes (142). YidD codes for a small peripheral membrane protein (85 amino acids, 9.4 kDa) that is supposed to be associated with the inner membrane via a putative N-terminal amphipathic helix. YidD is not an essential protein unlike YidC (142). However, a reduced membrane insertion efficiency for the YidC substrates, including M13, CyoA and F0, has been observed in yidD-null cells, pointing out a functional relationship between YidD and YidC (142). In vitro cross-linking study revealed that YidD is in contact with a nascent FtsQ in the proximity to the Sec translocon. In addition, a tight interaction of YidD with SecY and NuoK has been observed (Geng et al., 2015, submitted). Remarkably, membrane insertion of NuoK, which is YidC-SecYEG-dependent, is stimulated by YidD. The data suggest that YidD serves as a new but non-essential component in SecYEG-YidC-mediated membrane insertion.

5. Concluding remarks

Membrane biogenesis of the large protein complexes involved in energy transduction, respiration and light harvesting is mediated by the YidC/Oxa1/Alb3 protein family that is conserved in bacteria and eukaryotes. Over the last decade, major progress has been made to understand the molecular mechanism of the YidC/Oxa1/Alb3 machinery in the membrane biogenesis. The breakthrough has been the determination of the high-resolution crystal structures of YidC from E. coli (3.2 Å) and B. halodurans (2.4 Å), which shed light on the mechanism by which single-spanning membrane proteins are inserted into the membrane by the YidC insertases. However, the exact mechanism of YidC functioning in the membrane insertion of multi-spanning membrane proteins and folding remains elusive. Under physiological conditions, only ribosomes carrying the newly synthesized substrates can be tethered to YidC, implying specific dynamic interactions between YidC and the translating ribosome.
However, how the SRP targeting pathway discriminates between YidC-only and Sec-translocon dependent IMPs is unknown, nor is it fully elucidated what determines that IMPs require YidC for insertion. These are questions that can be resolved by future biochemical studies.

6. Scope of the thesis

The scope of the thesis is to determine the functional domains of YidC homologs in *B. subtilis* (SpoIIIJ and YqjG) and the *E. coli* (YidC) that contribute to substrates specificity and ribosome binding. In addition, the function of YidD, a proposed accessory protein in co-translational insertion was investigated.

Chapter 1 provides a review on our current understanding of the structure and function of the membrane protein insertase YidC.

Chapter 2 describes an analysis of the specific regions of *B. subtilis* SpoIIIJ that characterize its sporulation-specific function. SpoIIIJ is essential for sporulation, which could not be complemented by YqjG. A series of SpoIIIJ/YqjG chimeras were constructed by systematically swapping the TMSs of SpoIIIJ with those of YqjG and vice versa. The chimeras exhibited similar ability in restoring the growth defect of a *spoIIIJ yqjG* double deletion strain, confirming the *in vivo* activity. Distinct sporulation efficiencies were observed for a *spoIIIJ* deletion strain harboring the different chimeras, which highlighted the essential role of a domain comprising TMS2 and its flanking loops in sporulation. Furthermore, TMS2 functions in conjunction with the TMS1 and -3 of SpoIIIJ to fully support the sporulation specific function.

In Chapter 3, the domains of the *E. coli* YidC essential for ribosome binding and insertion activity are analyzed. Sequential deletions were introduced into the two cytosolic loops of YidC to generate a series of YidC variants. Growth complementation of the *yidC* depletion strain was employed to analyze the activity of YidC variants in cell viability. To monitor ribosome binding, a highly sensitive technique of FCS was applied with the fluorescently labeled YidC proteins in a native-like environment (termed nanodiscs). No obvious correlation was obtained between YidC insertion activity and ribosome binding. Remarkably, combining the deletions within the C2 loop and the C-terminus of YidC had a detrimental effect on ribosome binding and insertion activity. We speculated that the concomitant depletion of the C2 loop and the C-terminus of YidC lead to a conformational alteration or invalid interaction.
between YidC and RNCs that abolishes substrates insertion.

**Chapter 4** investigates the role of YidD in co-translational insertion pathway. Deletion of the *yidD* gene resulted in a reduction of the proton motive force and a weak overexpression of the stress protein PspA. YidD was co-purified with SecY and the NADH dehydrogenase subunit NuoK. Membrane insertion of NuoK, requiring both SecYEG and YidC, was stimulated by YidD, whereas membrane insertion of subunit c of the *F*<sub>1</sub>*F*<sub>0</sub> ATPase, which is YidC-dependent, was not affected by YidD. Our data suggest that YidD is a new component of the SecYEG-YidC translocase.

Finally, in **Chapter 5**, the findings reported in this thesis are summarized with a future perspective on mechanistic studies of YidC-mediated membrane insertion.

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