The bacterial membrane insertase YidC:

In vivo studies of substrate binding and membrane insertion

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To myself, for hanging in there

Contents

List of publications included in this thesis

I Christian Klenner, Jijun Yuan, Ross E. Dalbey and Andreas Kuhn (2008)
The Pf3 coat protein contacts TM1 and TM3 of YidC during membrane biogenesis.
FEBS Lett ***582****, 3967-72*

II Christian Klenner and Andreas Kuhn (2012)
Dynamic disulfide scanning of the membrane-inserting Pf3 coat protein reveals multiple YidC substrate contacts.
J Biol Chem ***287****, 3769-76*

Other publications

III Lu Zhu, Christian Klenner, Andreas Kuhn and Ross E. Dalbey (2012)
Both YidC and SecYEG are required for translocation of the periplasmic loops 1 and 2 of the multispanning membrane protein TatC.
J Mol Biol ***424****, 354-67.*

Abstract

Membrane proteins play a key role in many cellular processes. As a prerequisite for proper function, these proteins have to be inserted into biological membranes. The insertion process involves highly conserved translocation machineries – the translocons. In bacteria, YidC acts in cooperation with the Sec translocon, the main insertion site for membrane proteins. In addition, YidC can function independently of the Sec translocon, e.g. facilitating biogenesis of respiratory complexes and the F_1F_0-ATPase. This is most likely a reason why YidC is an essential protein in *Escherichia coli*.

At the time this project was initiated high resolution 3D structures of YidC were limited to the non-functional large periplasmic loop and it was largely unclear how YidC substrates are inserted into the membrane. This thesis aims to identify YidC-substrate contacts during membrane biogenesis and to investigate the molecular mechanism underlying YidC mediated insertion.

For capturing protein-protein interactions we have established an *in vivo* cross-linking assay using a set of single cysteine mutants of YidC and the inserting small phage protein Pf3 coat representing a model substrate of the YidC insertion pathway. We found that YidC contacts Pf3 coat protein with various regions of the conserved transmembrane (TM) core domains, which had been shown to be critical for function. An expressed Pf3 mutant with a defect in membrane insertion was unable to contact TM residues of YidC facing the periplasmic leaflet, whereas residues at the cytoplasmic leaflet were still contacted. We therefore suggest that the YidC mediated insertion is a dynamic process with early binding followed by the translocation and insertion of substrate proteins.

Abbreviations

Adenosine triphosphate	ATP
Amino acids	AAs
Amino (terminus)	N
Alb	Albino
Alkaline phosphatase	PhoA
Angstrom	Å
Arabidopsis thaliana	*A. thaliana*
Bacillus halodurans	*B. halodurans*
Bacillus subtilis	*B. subtilis*
Blue native	BN
Cardiolipin	CL
Carboxy (terminus)	C
Cytochrome c oxidase	Cox
Cytoplasmic domain	C
Debye–Waller factor	B-factor
Deoxyribonucleic acid	DNA
Electron microscopy	EM
Endoplasmic reticulum	ER
Escherichia coli	*E. coli*
Fluorescence cross-correlation spectroscopy	FCCS
Green fluorescent protein	GFP
Gene product	gp
Guanosine triphosphate	GTP
Hemoglobin protease	Hbp
Inner membrane	IM
Inner membrane proteins	IMPs
Kilodalton	kDa
Lactose permease	LacY
Light-harvesting chlorophyll-binding proteins	LHCPs
Membrane protein	MP
Messenger ribonucleic acid	mRNA
Micrometer	µm
Nanometer	nm
Nicotinamide adenine dinucleotide (oxidized)	NADH
Outer membrane	OM
Oxidase assembly 1	Oxa1
Oxidase assembly 2	Oxa2
Periplasmic domain	P
Phage shock protein A	PspA
Phosphatidylcholine	PC
Phoshatidylethanolamine	PE
Phosphatidylglycerol	PG
Phosphatidylinositol	PI
Polyacrylamide gel electrophoresis	PAGE
Proton motive force	pmf
Ribosomal ribonucleic acid	rRNA
Ribosome nascent chain	RNC
Saccharomyces cerevisiae	*S. cerevisiae*
Secretory	Sec
Signal recognition particle	SRP
Single-stranded deoxyribonucleic acid	ssDNA
Stimulated emission depletion	STED
Tail-anchored membrane proteins	TAMPs
Three-dimensional	3D
Translocase of the inner membrane	TIM23
Trigger factor	TF
Twin arginine translocation	Tat
Two-dimensional	2D
Transmembrane	TM

Amino acids	*3 - letters*	*1 - letter*
Alanine	Ala	A
Asparagine	Asn	N
Arginine	Arg	R
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenyalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

General Introduction

1.1 BIOLOGICAL MEMBRANES

All living cells are surrounded by at least one membrane. A cell is defined as a living unit and is separated thereby from its neighboring cellular environment. In eukaryotic cells, membranes of the endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, chloroplasts and other membrane enclosed organelles specify characteristic differences in content and function of these diverse cell organelles and the cytoplasm. In bacteria a distinction is made between Gram-positive and Gram-negative bacteria based on membrane architecture. In contrast to Gram-positive bacteria, which have a single membrane, Gram-negative bacteria possess two membranes, an inner and an outer membrane.

1.1.1 FEATURES OF BIOLOGICAL MEMBRANES

Even though each membrane exhibits unique functions, most membranes show correlated major features: (i) building of physical borders to maintain specific compositions and efficient control of biochemical processes in different membrane enclosed organelles, (ii) transport of a restricted class of molecules through the lipid bilayer – this is called the semipermeable character of membranes, (iii) acting as interfaces to transduce signals between different cell compartments, and (iv) maintaining essential cellular functions by providing an ideal environment for the activity of enzymes, ion pumps or receptors which are linked to functions (ii) and (iii).

Biological membranes are mainly consisting of amphipathic lipids and proteins with a variable amount of carbohydrates. They are represented by bilayers of lipids, which are organized in approximately 60 Ångstrom (Å) leaflets with their polar head groups facing the two surfaces (approximately 15 Å each) and the nonpolar hydrocarbon chains forming the hydrophobic core region (30 Å) (Figure 1.1). The chemistry of the

membrane lipids ensures the structure of the lipid bilayers. While mostly two long fatty acid hydrocarbon chains have to be separated from water, the polar head groups interact with the hydrogen-bonding network of the water. Thus, the formation of lipid bilayers from amphipathic lipids in aqueous solutions is driven by the hydrophobic effect and occurs spontaneously (TANFORD, 1973).

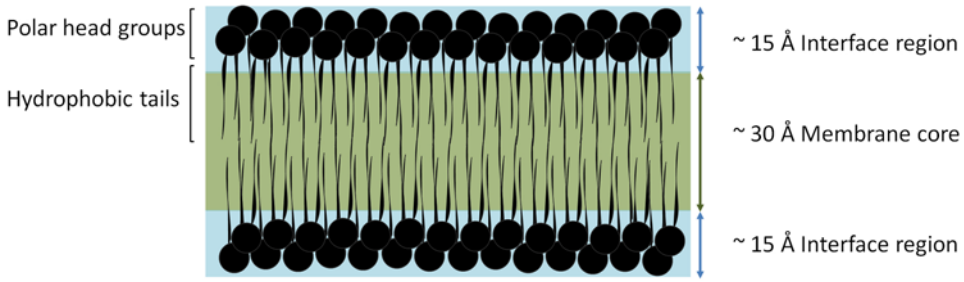

FIGURE 1.1 **Schematic representation of a lipid bilayer.** Black spheres represent the polar head groups and black sticks represent the hydrophobic tails. The thickness of a typical membrane consisting of a phospholipid bilayer is indicated.

The given description of the characteristics and the functions of biological membranes are based on the famous paper by Singer and Nicholson published in the year 1972 entitled *"The fluid mosaic model of the structure of cell membranes".* They established the first model which is applicable to most biological membranes, and revealed the membrane to be of crowded, heterogeneous, asymmetrical and dynamic nature consisting of integral or peripheral proteins in a fluid matrix of lipids (SINGER & NICOLSON, 1972). Forty years later, the basic principles of the model are still valid but experimental data have drawn a new picture of membranes as mosaic structures with heterogeneous proteolipid areas containing proteins organized mostly in an oligomeric state that form a bilayer of various thickness and composition (ENGELMAN, 2005; NICOLSON, 2014).

1.1.2 LIPIDS AND MEMBRANE COMPOSITION OF BACTERIA AND EUKARYOTES

Phospholipids (also referred to as glycerophospholipids), glycolipids and sterols are the major classes of membrane lipids. The diversity of different membrane lipids in the three domains of life is impressive. However, in a simple organism such as *Escherichia coli* (*E. coli*), the number of individual phospholipid species is in the order of hundreds. In more complex eukaryotes, with a higher diversity of phospholipids and fatty acids, the number is in the order of thousands.

Biosynthesis of membrane lipids occurs at the cytoplasmic leaflet of the inner membrane (IM) in bacteria and mainly at the ER in eukaryotes by a set of catalytic membrane bound or cytosolic enzymes. The expression of coding genes, involved in initial carboxylation steps (*acc* genes), fatty acid biogenesis (*fab* genes) and phospholipid synthesis (*pls* genes) is strictly controlled. For example, many Gram-positives express FapR, a global transcriptional factor, to regulate all the genes involved in lipid metabolism (SCHUJMAN et al., 2003). Membrane lipid homeostasis is challenging for all organisms and they have to adjust lipid composition in response to a changing environment constantly.

The composition of different membrane systems in archaea, bacteria and eukarya varies tremendously. Archaeal membranes and cell surfaces are structurally incomparable to other membranes and consist of unique mono- or bilayer forming lipids. In *E. coli*, a well-known model organism and representative of Gram-negative bacteria, the phospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL, also called diphosphatidylglycerol) are the major head groups of membrane lipids (AMES, 1968). The backbone is occupied predominantly by palmitic acid (16:0) and monounsaturated fatty acids palmitoleic (16:1) and *cis*-vaccenic acids (18:1). The composition of phospholipid head groups in the inner leaflet of the outer membrane (OM) and the IM in *E. coli* is identical: 70 to 80 % of PE, 20 to 25 % of PG and 5 % or less of CL (DOWHAN, 1997). In the outer monolayer of the OM another type of phospholipid unique to Gram-negative bacteria is present: the glucosamine- and lipid A-based lipopolysaccharide also known as endotoxin because of its toxic effects during Gram-negative infections (RAETZ, 1990). Many Gram-positive bacteria lack the zwitterionic PE but contain derivatives of anionic PG which are either zwitterionic or net positively charged.

The mass ratio of protein to lipid in bacterial membranes is approximately 3:1. For comparison, the simplest biological membrane system - vertebrate myelin - has a ratio of approximately 1:4, because myelin mainly acts as an insulator with no enzymatic function (GUIDOTTI, 1972).

In eukaryotic cells, the ER is the main organelle involved in phospholipid and cholesterol synthesis. Other places for phospholipid synthesis are mitochondria and the Golgi apparatus. Late endosomes and the plasma membrane are responsible for synthesis of

minor phospholipids like phosphatidylinositol (PI) derivates and of sphingosines. Both are signaling lipids involved in signal transduction. With respect to the cellular function, each organellar membrane is packed with various lipids. The ER membrane is loosely packed with phosphatidylcholine (PC), PE and PI (VAN MEER et al., 2008) to allow proper function as an organelle for insertion and transport of newly synthesized proteins and lipids. Sterols synthesized at the ER are rapidly transported to the plasma membrane and to endosomes. Together with sphingolipids, cholesterol is then packed at high density in the plasma and endosomal membranes to resist mechanical stress and osmotic pressure. The lipid composition of mitochondrial IMs is similar to their bacterial ancestors (VAN MEER & DE KROON, 2011; HORVATH & DAUM, 2013). They are enriched of PC, PE and CL and synthesize PG as a precursor of CL. The protein/lipid ratio of the IM of mitochondria is 4:1 and thus very high (LUCKEY, 2008) and comparable to the ratio found in bacteria. In general, a high enzymatic activity of an organelle correlates with high protein content. Thus, rough ER, chloroplasts and nuclear membranes have higher amounts of protein than for example myelin, smooth ER and Golgi membranes.

1.2 MEMBRANE PROTEINS

Representative biological membranes contain many types of proteins. These membrane proteins (MPs) maintain essential cellular functions and processes such as signaling, biogenesis, ion and nutrient transport and metabolism. A common distinction is made between peripheral (extrinsic) and integral (intrinsic) membrane proteins (SINGER & NICOLSON, 1972). Members of a third type of membrane proteins are called amphitropic proteins (JOHNSON & CORNELL, 1999).

1.2.1 PERIPHERAL MEMBRANE PROTEINS

Peripheral membrane proteins are mainly water-soluble proteins attached to the membrane surface by electrostatic interactions or hydrogen bonds between either surface exposed portions of integral membrane proteins, anionic phospholipid head groups or both (Figure 1.2). During membrane preparation it is easy to separate peripheral proteins clearly from the membrane fraction by altering pH or buffer salt

concentration. An extensive systematic analysis of the *E. coli* peripheral IM proteome by efficient subfractionation experiments revealed that approximately 17 % of the basal proteome are peripheral IM proteins (PAPANASTASIOU, 2013).

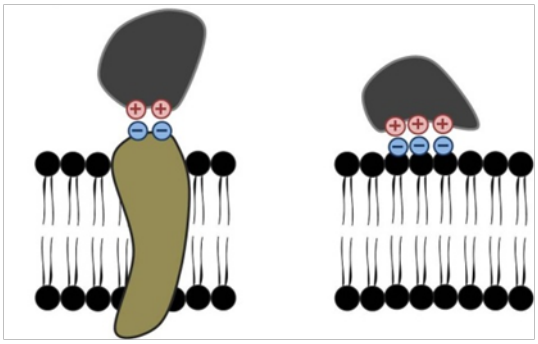

FIGURE 1.2 Electrostatic interactions of peripheral proteins with integral proteins (left) or anionic phospholipids (right).

A typical example of a peripheral protein is the human cytochrome c, a 12 kilodalton (kDa) small heme c containing protein of the intermembrane space of mitochondria. It binds to cytochrome c oxidase mainly via a cluster of carboxy-terminal (C-terminal) arginine and lysine residues (NICHOLLS, 1974). Furthermore, electrostatic interactions with anionic phospholipids have been shown for cytochrome c in circular dichroism and surface plasmon resonance studies (DE JONG & DE KRUIJFF, 1990; STEPANOV et al., 2009). In bacteria, the SRP receptor FtsY is attached to the membrane surface via anionic phospholipids (DE LEEUW et al., 2000) and interacts directly with the integral multi subunit Sec translocon (ANGELINI et al., 2005).

1.2.2 AMPHITROPIC PROTEINS

The class of amphitropic proteins is a special group of peripheral proteins. These proteins have two obvious localizations: one form is located in an aqueous environment and one form is located at the membrane (JOHNSON & CORNELL, 1999). The reversible binding to the membrane regulates the function of these proteins in various cellular processes. Three different principles explain how interactions with the lipid bilayer are achieved: (i) binding by 'lipid clamps' structures; (ii) attachment of lipid anchors for transient membrane insertion, often combined with exposed positive residues for electrostatic interactions; (iii) partitioning of an amphipathic alpha-helix (α-helix) into

the membrane bilayer. The bacterial protein SecA is a famous representative of amphitropic membrane proteins, as its ATPase activity is regulated by anionic phospholipid binding (LILL et al., 1990). Further cellular functions of the remarkable nanomachine will be discussed later.

1.2.3 INTEGRAL MEMBRANE PROTEINS

By far the largest class of membrane proteins is the class of integral (intrinsic) membrane proteins. They mediate plenty of cellular processes in transport, metabolism, biogenesis and signalling. In contrast to peripheral membrane proteins, integral ones are embedded firmly into the bilayer by hydrophobic interactions between the lipid hydrocarbon core and hydrophobic stretches of the proteins, and can only be removed by the use of detergents (amphipathic surfactants that disrupt the interactions between the hydrophobic domain of the protein and the lipid hydrocarbon core).

There are two model-like types of integral MPs which differ in their secondary structure and localization (Figure 1.3). Data from known structures clearly illustrate that the most common structural motifs in the transmembrane part of integral membrane proteins are α-helical bundles and β-barrels. During biogenesis α-helical MPs are folded into more or less complex bundles, predominantly perpendicular to the membrane. In general, they have longer and more hydrophobic transmembrane (TM) segments than the β-barrels. Transmembrane β-barrels are formed by up to 22 antiparallel, tilted β-strands (like e.g. in the iron-siderophore transporter FhuA). The first and last β-strands close the barrel upon interaction. The differences in those two structural motifs are dictated by the encoded sequence and the biogenesis of proteins in the lipid bilayer. Here it is important to consider that a polypeptide chain obtains the most stable conformation by formation of interchain peptide backbone hydrogen bonds (H-bonds) in the lipid bilayer (VON HEIJNE, 1994).

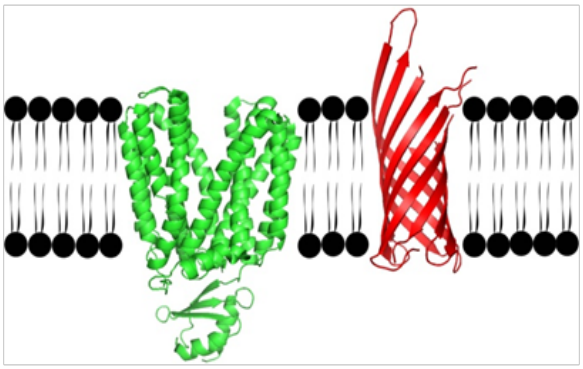

FIGURE 1.3 **Two known structural classes of integral membrane proteins**: ***α*-helical bundle** (in green; structure of the YajR transporter, PDB 3WDO), **β-barrel** (in red; structure of the OmpA membrane domain, PDB 1QJP)

Transmembrane β-barrels are commonly found in the OM of Gram-negative bacteria, mitochondria and chloroplasts (SCHULZ, 2000). The functions are various, including transport by diffusion (e. g. the trimeric porin proteins OmpF and LamB), bacterial adhesion (OmpX) and bacteriocin release (Phospholipase A). A characteristic feature of trans-membrane segments of β-barrel proteins is a stretch of typically 10 altering hydrophilic and hydrophobic amino acids (AAs) in the primary sequence. After secondary structure formation during barrel biogenesis, the hydrophobic residues face out towards the surrounding membrane lipids – the inner polar core is coated with hydrophilic residues (COWAN et al., 1992). Analyses of genomic sequence data from Gram-negative bacteria with statistical algorithms unmask approximately 2 to 3 % of the proteome as transmembrane β-barrel proteins (FREEMAN JR & WIMLEY, 2012). The present thesis focuses on the biogenesis of α-helical inner membrane proteins (IMPs), therefore the biogenesis of β-barrel proteins will not be further discussed.

α-helical membrane proteins are much more abundant than β-barrels. In bacteria 20 – 30 % of all genes are coding IMPs (WALLIN & VON HEIJNE, 1998; KROGH et al. 2001). Although the role of an increasing number of IMPs is solved, the function of about one third remains still unknown (DALEY et al. 2005). IMPs fulfill diverse and essential functions and the relevance for all living cells is not only due to their sheer abundance. IMPs are indispensable for transport, metabolism, signalling and biogenesis.

Transmembrane α-helices were thought to be typically oriented more or less perpendicularly to the plane of the membrane, and to span the membrane 1 to 18 times in *E. coli* (DALEY et al. 2005). Today it becomes obvious that membrane-embedded helices can vary in length, can contain bends and distortions, can be kinked or

interrupted in the middle of the membrane and even can span only a part of the membrane and then turn back (VON HEIJNE, 2006). The membrane embedded portion of IMPs consists of helical stretches with 15 to 30 largely hydrophobic AAs to span the hydrophobic core region of approximately 30 Å. Interestingly, charged AAs like lysine, arginine, aspartic acid and glutamic acid are frequently found within membrane helices although this is energetically not favorable. Often but not necessarily, such polar AAs within hydrophobic domains have a central role in protein function.

1.2.4 PROTEIN-LIPID INTERACTION

The lipid bilayer does not only function as a diffusion barrier and a simple matrix for integral or associated membrane proteins. Although bilayer forming and non-bilayer forming lipids (such as PE and CL, respectively) have no catalytic function, they can affect insertion, folding and assembly of MPs enormously. Patchy microdomains within the bilayer, based on defined lipid species compositions, enable the organization of large functional protein complexes.

In general, three types of binding modes for lipid interactions with MPs can be distinguished: (i) an annular shell of lipids bound to the protein surface, (ii) non-annular surface lipids immersed in cavities and clefts of the protein surface – this is found in multi-subunit complexes, (iii) lipids residing within a membrane protein or a membrane protein complex (PALSDOTTIR & HUNTE, 2004).

Specific protein–lipid interactions depend on the chemical and structural architecture of lipids. Protein function and membrane integrity also depend on common properties of lipids like self-association, shape and/or fluidity, as many membrane proteins undergo conformational changes during activity. For example, the MscL protein is allowed to properly open its large water filled pore only in response to lipid bilayer deformations, because the opening process needs transmembrane helical movements within the protein structure (PEROZO et al., 2002). Frequently, partial delipidation of membranes leads to a decrease in protein activity (DOWHAN, 1997). The proper function of some proteins strictly depends on the interaction with defined phospholipid species. For example the function of cytochrome bc1 complex is strongly coupled to a firm association of cardiolipin (SCHÄGGER et al., 1990; GOMEZ JR & ROBINSON, 1999). It has been shown *in vitro* that anionic PG and non-bilayer lipids stimulate protein

translocation mediated by the Sec translocon (VAN DER DOES et al., 2000). In addition, cardiolipin tightly associated with the Sec translocon, promotes an efficient binding of SecA and stimulates ATP hydrolysis (GOLD et al., 2010).

1.3 BIOGENESIS OF BACTERIAL α-HELICAL MEMBRANE PROTEINS

The biogenesis of a membrane protein can be divided into a few distinct steps (Figure 1.4). After gene transcription, the messenger ribonucleic acid (mRNA) is decoded by ribosomes in the cytoplasm. Then, the polypeptide is targeted to the cytoplasmic membrane and inserted into the membrane. This occurs co-translationally in most cases. The final destination of each protein is in general encoded in the amino-terminal (N-terminal) sequence of the nascent amino acid chain. The membrane insertion is catalyzed by the interaction of inserting membrane proteins with the Sec translocon or the YidC membrane insertase, or occurs spontaneously for very small hydrophobic proteins. During the membrane insertion process the topology is determined and a membrane protein adopts its secondary structure. After or during insertion the protein begins to fold properly into its native conformation. Following folding, many α-helical membrane proteins interact with other membrane or soluble proteins to form functional multi-subunit complexes.

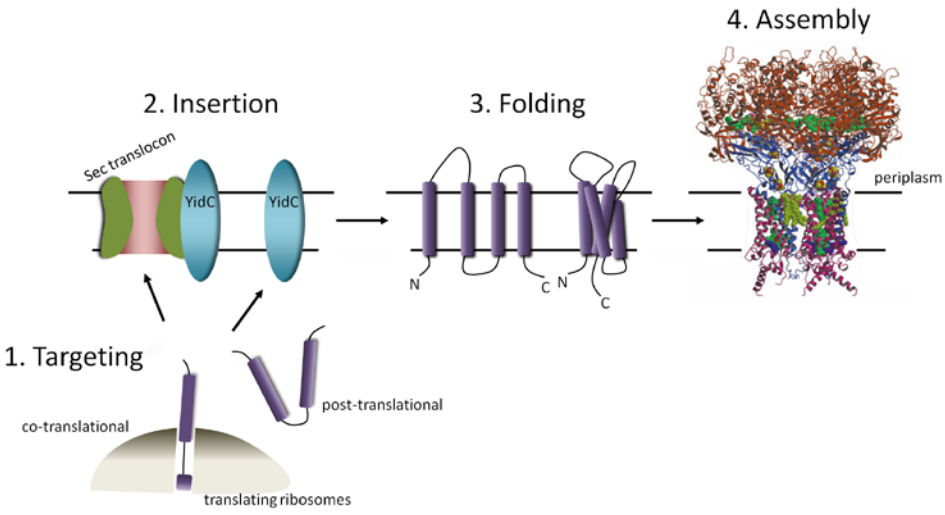

FIGURE 1.4 **Biogenesis of α-helical membrane proteins in *E. coli*** (see text for details).

1.3.1 PROTEIN TARGETING: HOW PROTEINS FIND THEIR FINAL DESTINATION

A challenging task for every cell is to ensure the correct transport of newly synthesized proteins to their final destination. Günter Blobel discovered in the 1970s that proteins carry discrete sequences, named signal sequences or topogenic sequences (BLOBEL & SABATINI, 1971; BLOBEL & DOBBERSTEIN, 1975; BLOBEL, 1980). In general, signal sequences are attachments at the N-terminus of the polypeptide chain that are decoded by cytoplasmic or membrane bound receptor proteins. In concert with different translocons and soluble or membrane bound protein factors, these targeting sequences pave the way to sort various polypeptide chains from each other. In bacteria, newly synthesized proteins need to be correctly localized and inserted into the IM and need to be exported to the extracellular place or, for Gram-negative, to the periplasmic space and the outer membrane.

In *E. coli* two major targeting pathways rule the direction of proteins: (i) post-translational pathways, in which the majority of periplasmic, OM and secretory proteins are targeted to the cytoplasmic membrane after protein synthesis, and (ii) the co-translational pathway, in which most α-helical IMPs are targeted to the membrane during the ongoing synthesis by the ribosome (SARAOGI & SHAN, 2013). In the following, the targeting of proteins destined for crossing the IM will be discussed briefly, and then the targeting of IMPs will be explained in more detail.

1.3.1.1 The signal sequence of secretory preproteins

Proteins, which are synthesized as preproteins (or precursors) and destined to locate in the periplasm, at the outer membrane or extracellular (in the following referred to as secretory proteins), usually have a cleavable signal sequence with a typical size of approximately 20 to 30 residues. The signal sequence can be divided into three different domains: (i) the 'N-domain' with a positive net charge, (ii) an 'H-domain' of approximately 7 to 13 mainly hydrophobic residues, and (iii) the slightly polar 'C-domain' that contains the cleavage site for the signal peptidase (VON HEIJNE, 1985). After the preprotein is translocated across the membrane, it is processed into its mature form by cleavage of the signal sequence by an externally signal peptidase (PAETZEL et al., 2002).

1.3.1.2 The post-translational SecB-pathway

In bacteria, many secretory proteins as well as periplasmic and outer membrane proteins are targeted to the cytoplasmic membrane post-translationally by SecB and are mostly transported across the membrane via the Sec translocon. Once the signal sequence exits the tunnel of a translating ribosome, a cytoplasmic chaperone, called trigger factor, conducts nascent preproteins into the post-translationally SecB pathway by preventing the interaction between the signal recognition particle (SRP) and the signal peptide (BECK et al., 2000). The molecular chaperone SecB captures nascent preproteins and keeps them in a loosely folded and non-aggregated state by binding the mature region of the preprotein (HARTL et al., 1990). SecB then targets the translocation-competent protein to the SecA ATPase which is tightly associated with the Sec translocon at the cytoplasmic face of the inner membrane (HARDY & RANDALL, 1993; FEKKES et al., 1998).

1.3.1.3 Twin-arginine translocation (Tat)-pathway: Targeting of folded proteins

Some secretory proteins are using an alternative targeting route. They are translocated in an entirely folded state via the Tat translocon, which is present in bacteria and the chloroplasts of plants. Tat substrate proteins typically bind metal cofactors and contain a specific signal sequence with the twin arginine (RR) consensus motif S-R-R-x-F-L-K located in the 'N-domain' (BERKS, 1996). Presumably cytosolic chaperones like DnaK (PEREZ-RODRIGUEZ et al., 2007) are involved in membrane targeting of Tat dependent proteins, but so far no specific targeting factor for Tat signal sequences (like SRP) has been uncovered. Interestingly, for a polytopic Rieske iron sulfur membrane protein from *Streptomyces coelicolor* expressed in *E. coli*, it has been shown that both the Sec and the Tat translocons are co-operating in membrane integration (KELLER et al., 2012). In contrast, some Tat substrates from *E. coli,* containing hydrophobic C-terminal transmembrane helices, are integrated into the lipid bilayer solely by the Tat translocon (HATZIXANTHIS et al., 2003).

1.3.1.4 SRP-pathway: The main pathway for inner membrane proteins

Most proteins that are intended to reside in the IM of *E. coli* are generally targeted to the membrane by the ubiquitous co-translational SRP-targeting pathway (DE GIER et al., 1996; ULBRANDT et al., 1997). The universally conserved ribonucleoprotein complex SRP

is present in all three kingdoms of life. Compared to its mammalian homolog, *E. coli* SRP is relatively simple and is comprised of the 48 kDa GTPase Ffh (a homolog of SRP54) and a small 4.5S RNA with 114 bases in length. Ffh consists of two domains: (i) the 'NG-domain' which is located at the N-terminus containing a GTP-binding site, and (ii) the C-terminal methionine rich 'M-domain'. The prokaryotic homolog of the SRP receptor SRα is the peripheral membrane protein FtsY. The receptor FtsY contains a similar NG domain like Ffh, which is located at the C-terminus, as well as a highly positively charged N-terminal 'A-domain'. Despite its simplicity, bacteria SRP can substitute eukaryotic homologs to promote efficient targeting to the ER membrane (BERNSTEIN et al., 1993).

The SRP targeting pathway is initiated when SRP detects an N-terminal sequence presented on polypeptide chains once they emerge from the translating ribosome (LUIRINK et al., 1992). Unlike signal sequences of secretory or Tat client proteins, signal sequences of IMPs which are recognized by SRP mostly have no cleavage site and no defined recognition feature. These targeting sequences are in general enhanced hydrophobic α-helical transmembrane domains and called signal anchor sequences. Proverbially, they anchor inserting membrane proteins permanently into the lipid bilayer with a $N_{in}C_{out}$ orientation. Thus, the first hydrophobic TM segment of an α-helical membrane protein often serves as a signal for membrane targeting (ZERIAL et al., 1986).

SRP binds the signal anchor sequence of a ribosome nascent chain (RNC) in a deep groove of the 'M domain', shaped by mainly hydrophobic residues, including the conserved methionine residues (KEENAN et al., 1998). Since a conserved domain of the 4.5S RNA binds to the 'M-domain' close to the groove, both protein and RNA most likely provide the signal sequence binding site. Structures obtained by cryo-electron microscopy (EM) of an RNC complex with *E. coli* SRP show that the distinct presentation of the signal sequence at the ribosomal tunnel exit allows an efficient slide of the signal sequence into the hydrophobic groove of the SRP 'M-domain' (SCHAFFITZEL et al., 2006). Therefore, specific binding of SRP to the ribosome tunnel exit is an important precondition. This is achieved by binding of the helical 'N-domain' to the ribosomal proteins L23 and L29 (HALIC et al., 2006; GU et al., 2003).

The membrane targeting of SRP bound RNC complexes (RNC-SRP) to the cytoplasmic side of the membrane is achieved by the interaction of the two 'NG domains' of SRP and its receptor. The receptor FtsY exists both in a soluble (LUIRINK et al., 1994) and a

membrane associated form, the latter most likely being preferred (ANGELINI et al., 2005). It has been shown that only membrane bound FtsY molecules are capable to promote dissociation of SRP from the RNC (LAM et al., 2010, VALENT et al., 1998). Altogether, the membrane association of FtsY is highly dynamic and it is discussed controversially, whether soluble FtsY is able to bind translating ribosomes aside the membrane (HERSKOVITS & BIBI, 2000). In order to finalize membrane targeting, the RNC-SRP-FtsY complex is anchored to the membrane by the positively charged α-helical 'A-domain' of FtsY (PARLITZ et al., 2007). At the membrane, GTP hydrolysis of both SRP and the receptor FtsY (in the GTPase G-domains of the proteins) results in the dissociation of the RNC-SRP-FtsY complex (CONNOLLY et al., 1991) and the recycling of SRP and FtsY into the cytosol for upcoming targeting events. In the most common cases the RNCs are released to the Sec translocon which facilitates the insertion of targeted membrane proteins alone or in cooperation with the YidC insertase (XIE & DALBEY 2008). Both genetic and structural studies showed clearly that basic residues of cytosolic SecY loops interact with ribosomal proteins L23 and L29 at the ribosomal tunnel exit (CHENG et al., 2005; MENETRET et al., 2007; BECKMANN et al., 2001; FRAUENFELD et al., 2011). As mentioned above, SRP binds to the ribosomal proteins L23 and L29 during the targeting process. Thus, a stable RNC-SecYEG complex formation requires the detachment of SRP from the RNC. As the translation resumes on Sec-associated ribosomes, the nascent polypeptide chain slips into the aqueous translocation channel or directly into the lateral gate region. During translocation, transmembrane segments exit the channel laterally into the lipid bilayer (VAN DEN BERG et al., 2004).

Besides the Sec translocon, the YidC insertase receives a small number of SRP substrates and mediates their membrane insertion. This has been shown for the mechanosensitive channel protein MscL and - to a certain degree - for F_oc, the subunit c of the ATPase (FACEY et al., 2007; VAN BLOOIS et al., 2004; YI et al., 2004). In addition, it has been shown that Ffh and FtsY directly contact YidC (WELTE et al., 2012).

The depletion of SRP (Ffh) in *E. coli* leads to global kinetic defects in the biogenesis and localization of IMPs, resulting in increased protein aggregation in the cytoplasm and finally to cell death. To overcome the protein aggregation, the cells show a strong σ^{32} response which leads to upregulated expression levels of molecular chaperones in the cytoplasm (WICKSTRÖM et al., 2011a). Another study revealed that SRP depletion has no significant negative influence on the steady-state level or distribution of most inner

membrane proteins. On the other hand, SRP depletion leads to an immediate reduction of the proton motive force (pmf) (ZHANG et al., 2012). These observations show clearly that SRP is essential for global cell integrity, but they also suggest that alternative (SRP-independent) targeting pathways for inner membrane proteins exist in *E. coli*.

1.3.1.5 Non-classical targeting pathways

The affinity of ribosomes for the Sec translocon is highly conserved (PRINZ et al., 2000). Therefore, it is obvious that ribosomes might support co-translational targeting independently of SRP. In eukaryotes, ribosomes remain associated at the ER membrane after co-translational targeting and can be primed by an mRNA encoding a membrane protein (POTTER & NICCHITTA, 2002). In bacteria, it has been shown that mRNAs coding for inner membrane proteins are targeted to the membrane in a translation independent mechanism (NEVO-DINUR et al., 2011). Most likely there is a correlation between the uracil content and the localization of mRNAs, as mRNAs of membrane proteins have significantly higher uracil content (PRILUSKY & BIBI, 2009).

Tail-anchored membrane proteins (TAMPs) are a small, heterogeneous class of proteins in *E. coli* which are anchored to the membrane with a C-terminal transmembrane segment and consequently contain no N-terminally located hydrophobic signal sequence (BORGESE & RIGHI, 2010; CRANEY et al., 2011). Together with a few small < 50 AAs single spanning membrane proteins in *E. coli* the TAMPs have to be targeted and inserted into the membrane independently of SRP and the Sec translocon. Factors promoting targeting and insertion of TAMPs as found in eukaryotes (Get pathway) and archaea (ArsA homolog) have not been identified so far. The detailed mechanisms of the biogenesis of these proteins remain to be investigated. Recently it has been shown that the TAMP TssL, a component of the Typ VI secretion system of enteroaggregative *E. coli*, requires the membrane insertase YidC and with some limitations the molecular chaperone DnaK for membrane biogenesis, but not the Sec-system (SOUSSOULA & KUHN, unpublished data; ASCHTGEN et al., 2012).

M13 procoat protein, the coat protein of filamentous *E. coli* phage M13, is targeted to the membrane in a passive mode, without any targeting factors. Here, the targeting is promoted by electrostatic interactions between positively charged AAs at the C- and N-termini and the negatively charged head groups of membrane phospholipids (GALLUSSER

& KUHN, 1990). There is evidence that Pf3 coat protein of *Pseudomonas aeruginosa* phage Pf3 is targeted to the membrane in the same way, since two positively charged residues are present at the C-terminus of the protein.

The role of "classical" chaperones like Trigger factor (TF), DnaK and GroEL in an alternative targeting pathway is not clear despite their expression being up-regulated in cells depleted of SRP (WICKSTRÖM et al., 2011a; ZHANG et al., 2012). However, there is evidence from *in vitro* studies, that the chaperone GroEL might mediates post-translational targeting of the polytopic membrane protein lactose permease (LacY) and bacteriorhodopsin (BOCHKAREVA et al. 1996; DEATON et al., 2004).

1.3.2 TOPOLOGY OF MEMBRANE PROTEINS

A fundamental aspect in the biogenesis of membrane proteins is the question how a given polypeptide chain is oriented in the lipid environment during insertion. The topology of a membrane protein can be considered as a 2D representation of the protein and is defined by the number of transmembrane helices and the orientation of the N- and C-terminus relative to the lipid bilayer (Figure 1.5). Topology maps can be predicted theoretically by using algorithm based prediction programs or experimentally by using terminal tagging with alkaline phosphatase (PhoA) and green fluorescent protein (GFP) (DALEY et al., 2005).

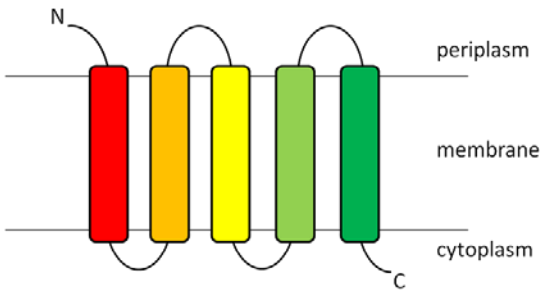

FIGURE 1.5 **Topology of a membrane protein** with five transmembrane helices (colored). The amino-terminus (N) is located in the periplasm (referred to as 'outside') and the carboxy-terminus (C) is located in the cytoplasm (referred to as 'inside'). The transmembrane helices (colored) are connected via short loops.

Well defined topogenic signals reside in the protein sequence. Topogenic signals are recognized and decoded by the translocation and insertion machineries after targeting

to the membrane (BLOBEL, 1980). The proper orientation of TM segments is an absolute precondition for effective folding into the native 3D conformation.

1.3.2.1 Topogenic signals define topology of membrane proteins

The variety of topogenic signals and the resulting topologies of different model proteins are summarized and illustrated in Figure 1.6. There are two classes of single-spanning membrane proteins (Figure 1.6A), commonly called type I and type II membrane proteins (VON HEIJNE & GAVEL, 1988). Type I membrane proteins contain a reverse signal anchor (also called type I signal anchor) which facilitates the translocation of the polar N-terminus and anchors the protein into the membrane with an $N_{outside}$-C_{inside} orientation. An example of such a protein is Pf3 coat (ROHRER & KUHN, 1990). Other type I proteins are synthesized as precursor proteins with a cleavable signal sequence and a stop transfer sequence as topogenic elements. The cleavage by signal peptidase after insertion results in type I orientation of the mature protein. For the phage protein M13 gp3, the signal sequence initiates translocation of the hydrophilic domain. The mature C-terminal transmembrane segment contains a stop transfer signal to terminate translocation. In contrast, M13 procoat protein (gp8) is inserted by a different mechanism, yet still adopting the same topology as gp3 does. Here, the insertion signals are located in the signal sequence and the membrane segment, both elements forming a topogenic element called 'helical hairpin' to translocate the polar domain (KUHN et al., 1986; ENGELMAN & STEITZ, 1981). Type II membrane proteins (with an N_{inside}-$C_{outside}$ orientation) possess an uncleaved signal anchor (or type II signal anchor) that initiates translocation of the C-terminus across the membrane. For example, the cell division protein FtsQ is a type II membrane protein (CARSON et al., 1991).

The topologies of double- and multi-spanning membrane proteins are conducted by discrete hydrophobic transmembrane segments and the interplay of the various topogenic signals described above. Examples for these complex membrane proteins are illustrated in Figure 1.6B and C.

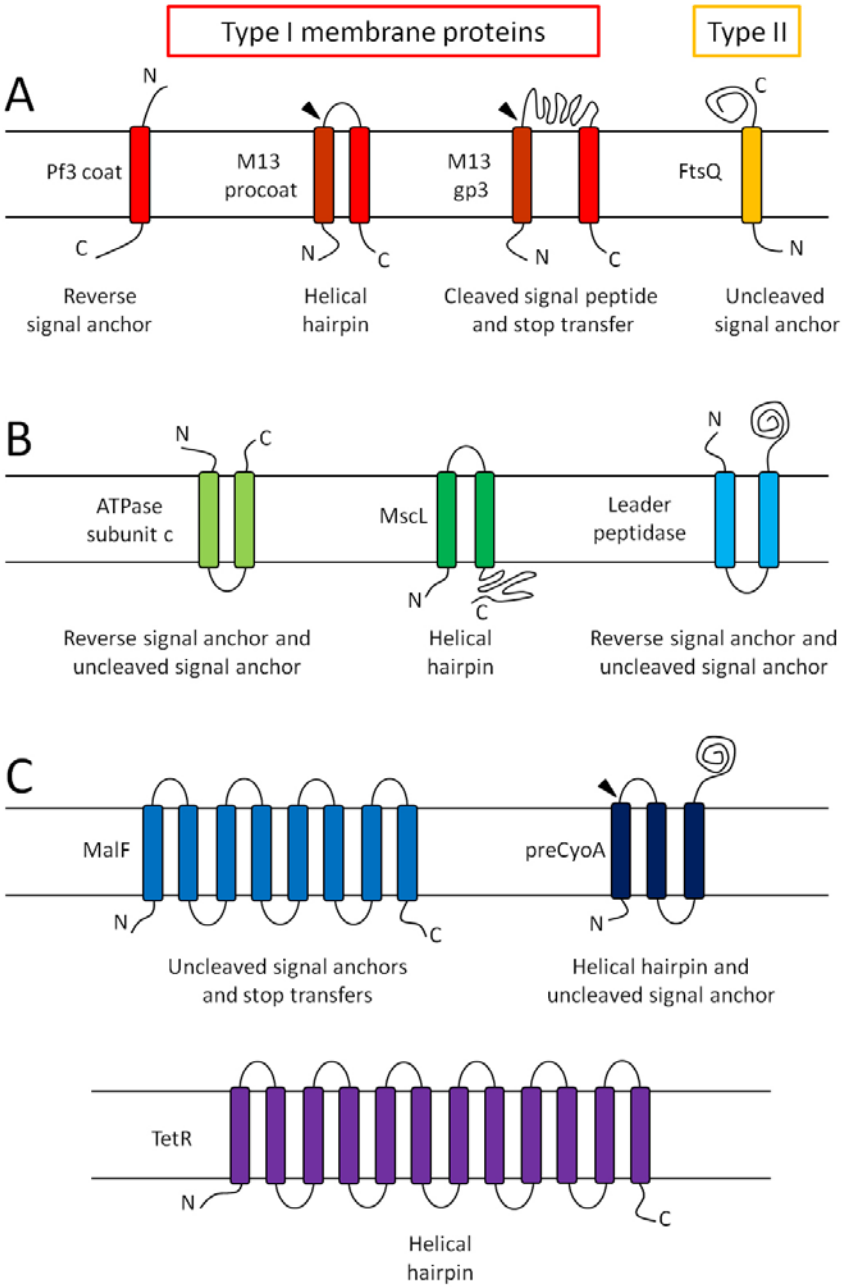

FIGURE 1.6 **Topology of membrane proteins and topogenic signals. (A)** Single-spanning membrane proteins of type I (Pf3 coat, M13 procoat and M13 gp3) and type II (FtsQ). **(B)** Double-spanning membrane proteins. **(C)** Multi-spanning membrane proteins. The arrowhead symbols indicate cleavage by signal peptidase after insertion.

The topogenesis of multi-spanning membrane proteins can be considered as the consecutive insertion of alternating start- and stop-transfer sequences or helical hairpin loops. In the simplest model the first transmembrane segment, i. e. the initial topogenic signal, defines the orientation of itself and the alternate orientation of following transmembrane segments. The insertion mechanism is not that strict for all multi-

spanning proteins, what has been shown for the MalF protein. Most likely the signals for proper topogenesis are located throughout the whole MalF protein, since depletion of the second transmembrane segment does not alter orientation of downstream segments (McGovern et al., 1991).

The hydrophobicity of transmembrane segments is crucial for their function in serving as topogenic sequences. Interestingly, sequence-comparison of the different hydrophobic topogenic sequences did not show any significant deviation in the AAs composition but a clear difference between the downstream and upstream polar flanking regions (von Heijne & Gavel, 1988).

1.3.2.2 The universal positive-inside rule

The positive-inside rule by Gunnar von Heijne is postulating that membrane protein topology is primarily determined by charged residues in cytoplasmic domains flanking hydrophobic transmembrane segments. In general, the positively charged residues arginine and lysine (Arg and Lys) are up to 4-times more prevalent in cytoplasmic domains compared to the 'outside' (von Heijne, 1986). The hypothesis derived from statistical analysis of *E. coli* membrane proteins was confirmed experimentally; showing that leader peptidase from *E. coli* (Figure 1.6B) reverses its topology when additional lysine residues are placed at the N-terminus (von Heijne, 1989). Genome-wide analysis of the membrane proteome in all domains of life revealed the universality of the positive-inside rule (Nilsson et al., 2005).

A comparable significant enrichment of negatively charged residues has not been detected in any extramembrane domains (Granseth et al., 2005; Nilsson et al., 2005). Yet, there is evidence that the negatively charged AAs glutamic acid or aspartic acid (Glu and Asp) also direct helix orientation (Nilsson et al., 1990; Delgado-Partin & Dalbey, 1998). For the biogenesis of Pf3 coat protein rather negatively charged residues than positively charged residues appear to be topogenic (Kiefer et al., 1997).

How discrete topological signals are interpreted by cell components is not fully understood. It is assumed that a complex interplay of the translocation and insertion machineries, helix characteristics and interactions within the protein, as well as the final membrane localization, which is mostly defined by the lipid composition, decode

topogenic signals. In the following I will describe factors that contribute to the determination of membrane topology.

1.3.2.3 Topological determinants

The lipid composition of the target membrane is one factor involved in guiding membrane topology. The negatively charged phospholipids PG and CL, being among the most abundant phospholipids in the inner *E. coli* membrane, direct positively charged protein domains to remain in the cytoplasm in accordance with the positive-inside rule (VAN KLOMPENBURG et al., 1997). In addition, neutral lipids like PE reduce the potential of negatively charged residues to serve as topogenic signals (DOWHAN & BOGDANOV, 2009). Alteration of lipid composition can lead to reversible orientations of multi-spanning membrane proteins such as lactose permease LacY and phenylalanine permease PheP (BOGDANOV et al., 2002; ZHANG et al., 2003).

The hydrophilic channel of the Sec translocon synchronizes the insertion of hydrophobic transmembrane segments and the translocation of flanking domains with different net charges to the 'outside'. In the yeast channel subunit Sec61p (homolog of prokaryotic SecY) conserved charged residues are located at the cytoplasmic (E382) and the 'outside' (R67, R74) facing end of the channel. These charged residues are suggested to contribute for orienting topogenic signals. Mutations of the charged residues affect the orientation of a model protein and reduce the influence of the positive-inside rule (GODER et al., 2004).

The pmf is another factor which presumably is involved in interpretation of topogenic signals in accordance with the positive-inside rule. In most bacteria, chemiosmosis leads to a positive outside and negative cytoplasm. This charge difference may prevent translocation of positively charged polypeptide domains and support translocation of negatively charged domains (CAO & DALBEY, 1994; ANDERSSON & HEIJNE, 1994; CAO et al., 1995; KIEFER et al., 1997). However, the retention of positively charged AAs cannot be exclusively determined by the pmf, since obligate acidophilic archaea with an inverted membrane potential show the same distribution of Arg and Lys residues in integral membrane proteins as neutrophilic bacteria (VAN DE VOSSENBERG, 1998). This fact underlines that electrostatic interaction between negatively charged phospholipid head

groups and positively charged residues is a much more relevant topological determinant.

Taken together, how and when topogenesis of membrane proteins is determined seems to be a complex event which is still very puzzling, because the majority of membrane proteins do not insert spontaneously into the membrane and require precise working translocases or insertases to orchestrate insertion.

1.3.3 INSERTION OF α-HELICAL MEMBRANE PROTEINS

Newly synthesized α-helical membrane proteins are primarily targeted to two different insertion sites: (i) to the Sec translocon/YidC and (ii) to the insertase YidC that is not associated with the Sec translocon. The Sec translocon is a protein-conducting channel present in all domains of life which is required for the translocation of secretory proteins and the insertion of α-helical membrane proteins. The membrane insertase YidC is also highly conserved but homologous proteins are missing in the phylum Crenarchaeota and in ER membranes (POHLSCHRÖDER et al., 2005). YidC promotes insertion, folding and assembly of α-helical membrane proteins both in cooperation with the Sec translocon and as an autonomous insertion site. YidC mediated biogenesis of membrane proteins will be discussed in detail in chapter 1.4 to underline the central significance of YidC for this thesis.

1.3.3.1 The Sec translocon: A protein-conducting channel for secretion and insertion

In bacteria, the Sec translocon is composed of a heterooligomeric complex of integral membrane proteins and the peripheral associated component SecA. The aqueous protein-conducting channel is formed by the essential core proteins SecY and SecE (homologs of the eukaryotic core components Sec61α and Sec61γ, respectively) and a distinct protein, SecG, which is not essential for cell viability (HANADA et al., 1994; HARTMANN et al., 1994). SecG shows no obvious homology to the corresponding β-subunits in eukaryotes and archaea. The accessory components SecDFYajC and YidC complete the membrane-embedded portion of the Sec translocon in some cases. As mentioned above, SecA is associated to the translocon as a peripheral membrane protein, which is, like SecY and SecE, essential for cell viability and has so far been only found in bacteria and chloroplasts (SARDIS & ECONOMOU, 2010). In addition, ribosomal

proteins and RNA contact the Sec translocon at multiple sites of cytosolic SecY domains (CHENG et al., 2005; KUHN et al., 2011; FRAUENFELD et al., 2011).

1.3.3.2 Accessory components of the Sec translocon and their function

SecD and SecF are polytopic membrane proteins with large periplasmic domains. Together with the single spanning YajC, SecDF forms a heterotrimeric complex associated to the SecYEG channel (DUONG & WICKNER, 1997). It is assumed, that SecDFYajC is not essential for the insertion, yet the complex most likely enhances the efficiency of this process (BRUNDAGE et al., 1990; HANADA et al., 1994; POGLIANO & BECKWITH, 1994; TSUKAZAKI et al., 2011). Structural analysis and biochemical data suggest that SecDF utilizes the pmf to complete the translocation of substrates (NOUWEN et al., 2005; TSUKAZAKI et al., 2011). YidC associates directly to SecYEG or to SecDF and functions in concert with the SecYEG channel (SCOTTI et al., 2000; NOUWEN & DRIESSEN; 2002; XIE et al., 2006; SACHELARU et al., 2013). Recently an intact and active complex of SecYEG, SecDFYajC and YidC (also known as the holotranslocon) was successfully overexpressed, purified and reconstituted for functional analysis of protein insertion and translocation (SCHULZE et al., 2014).

1.3.3.3 Structure of SecYEG

The X-ray crystallographic structure of the SecYEG/β complex from *Methanococcus jannashii* together with the cryo-EM structure of *E. coli* SecYEG, bound to an RNC, remarked a breakthrough in the field, providing first insights into the molecular organization and the detailed structure of the protein-conducting channel (VAN DEN BERG et al., 2004; MITRA et al., 2005). SecY consists of ten TM segments. The X-ray structure revealed that SecY forms an hourglass channel consisting of two clamshell-like domains of SecY TM domains 1-5 and TM domains 6-10, which are connected by a periplasmic loop between TM5 and TM6 (Figure 1.7). The center of the two halves represents the protein-conducting pore as two hydrophilic funnel-like cavities, which are open towards the cytoplasm and periplasm, respectively.

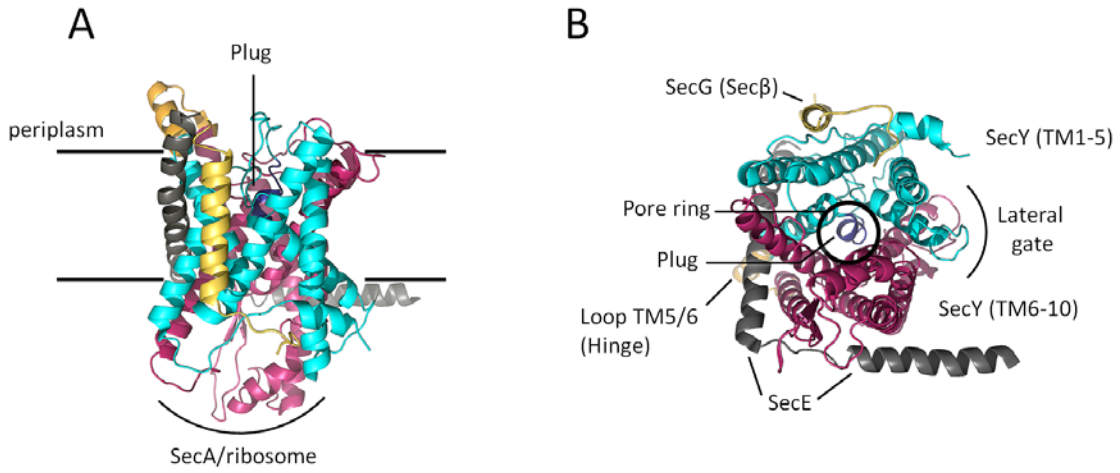

FIGURE 1.7 **X-ray structure of SecYEG(β) from archaea species *Methanococcus janaschii*** (PDB entry 1RHZ; VAN DEN BERG et al., 2004)**. (A)** Cross section of the membrane and **(B)** View from the cytosol to SecYEG(β). The hourglass-like structure of the protein-conducting channel is formed by SecY TM1-5 (cyan) and SecY TM6-10 (purple); both halves are connected by Loop TM5/6 (called the hinge domain, orange). SecE (grey) embraces the two channel forming halves of SecY. SecG (β) (golden yellow) is associated to the channel peripherally. On the periplasmic side the channel is blocked by helix 2a, the plug domain (blue). At the narrowest point of the channel hydrophobic residues form the pore ring (circle). The lateral gate is located at the front side of the clamshell-like SecY, as the potential exit side of transmembrane domains into the lipid bilayer. (Adapted from DRIESSEN & NOUWEN, 2008 with minor changes)

At the cytoplasmic side a ring of six hydrophobic residues of TM2b, 4, 7 and 10 block the channel at the narrowest point by forming a 'hydrophobic collar'(approximately 5 to 8 Å), called the pore ring. The channel pore is further blocked by the plug domain TM2a on the periplasmic side of the complex. The plug domain stabilizes the closed complex and prevents passage of small molecules through the channel (LI et al., 2007; PARK & RAPOPORT, 2011). During protein translocation, the plug moves out of the way to open the channel. Structural data and results of cross-link studies suggest that presumably both the signal sequences of secretory proteins and topogenic signal sequences of membrane proteins bind to TM2b, helix 8b and TM7 as molecular hairpins to trigger channel opening for the translocation of hydrophilic domains (VAN DEN BERG et al., 2004; FRAUENFELD et al., 2011; PARK et al., 2014; PLATH et al., 1998). The first crystal structure by Bert van den Berg also revealed that helices of both SecY halves form a so called lateral-gate to allow partitioning of TMs into the lipid bilayer through the front side of the channel (Figure 1.7B). First evidence for a lateral exit site came from early studies which have shown that hydrophobic signal anchor segments could be effectively cross-linked to lipids during early stages of membrane protein biogenesis (MARTOGLIO et al., 1995). The SecE protein, consisting of three TM domains in *E. coli*, most probably stabilizes the

channel by embracing the two SecY domains on the back side. SecG/β is located at the outside of the channel and shows only weak association to SecY.

It is still controversially discussed, whether SecY functions as a monomer or as a dimer to facilitate translocation and insertion. Evidence for a SecY dimer as the functional state were coming from studies in which both 'back-to-back' and 'front-to-front' dimeric forms of SecY in crystals have been observed (BREYTON et al., 2002; DALAL et al., 2012; MITRA et al., 2006). However, several crystal structures of SecY complexes from bacteria and archaea indicate that the active channel is formed by one copy of SecY (VAN DEN BERG et al. 2004; TSUKAZAKI et al., 2008; ZIMMER et al., 2008; EGEA & STROUD, 2010). In addition, cross-linking studies have shown that a single copy is sufficient to promote protein translocation, although both forms of SecY dimers have been found *in vivo* (PARK & RAPOPORT 2012). The latter is consistent with data that suggest that in a dimeric state of the translocon the nontranslocating complex can contribute to SecA binding and stimulation of its ATPase activity while the other copy promotes translocation of substrate proteins (OSBORNE & RAPOPORT, 2007; DALAL et al., 2012). Studies of protein secretion and insertion with the successfully reconstituted holotranslocon (with a monomeric stoichiometry) and results from single molecule experiments further suggest that a single complex is sufficient for function (SCHULZE et al., 2014; KEDROV et al., 2013). Recently obtained cryo-EM structures of ribosome bound Sec translocons in an active state revealed that a single SecYEG and Sec61 complex is associated with the ribosome during co-translational translocation (PARK et al., 2014; GOGALA et al., 2014). Both studies present sensational insights into conformational states within the active Sec translocon during translocation for the first time.

1.3.3.4 The Sec translocon in action: seemingly contrary functions

An exceptional feature of the Sec translocon is its capability to promote three essential cell processes: (i) the secretion of unfolded preproteins from the cytoplasm into the periplasm through the channel, (ii) the translocation of polar domains of membrane proteins across the membrane, and (iii) the insertion of hydrophobic transmembrane segments into the phospholipid bilayer (DRIESSEN & NOUWEN, 2008).

1.3.3.5 Translocation of secretory proteins across the membrane

The translocation of preproteins generally occurs in a post-translational route after targeting to the Sec translocon - mostly via the SecB pathway (HARTL et al., 1990). The energy for preprotein translocation is provided by ATP hydrolysis at SecA and by the pmf (DRIESSEN, 1992; VAN DALEN et al., 1999). The SecA protein is found predominantly in the cytoplasm, where it binds to translated SecB/preprotein complexes and at the membrane, where it is associated with the Sec translocon (HUBER et al., 2011; ZIMMER et al., 2008). For the translocation of a polypeptide chain SecA interacts with cytoplasmic loops of SecY and SecEG (MITRA et al., 2005; VAN DER SLUIS et al., 2006; ZIMMER et al., 2008; NAGAMORI et al., 2002). It is assumed, that the binding of SecA to SecYEG induces the plug displacement in order to open the channel (ZIMMER et al., 2008). The binding of ATP to SecA at the Sec translocon initiates translocation and allows the binding of the N-terminal signal sequence into the SecYEG channel. At next, multiple cycles of ATP binding and hydrolysis at SecA, that lead to repeated binding and release of the preprotein, cause the stepwise translocation of the polypeptide chain through the Sec translocon (ECONOMOU & WICKNER, 1994; VAN DER WOLK et al., 1997). The pmf is capable to drive translocation after ATP hydrolysis has lead to dissociation of the polypeptide chain from SecA (SCHIEBEL et al., 1991). How exactly the pmf acts as a driving force for translocation is not clear. It is conceivable that an electrophoretic mechanism drive the translocation of negatively charged residues. After translocation across the membrane has been completed, the preprotein is cleaved by a signal peptidase to its mature form, thus obtaining its native conformation (PAETZEL et al., 2002).

1.3.3.6 Insertion of membrane proteins into the lipid bilayer via the Sec translocon

The insertion of the majority of α-helical membrane proteins is catalyzed by the Sec translocon and occurs mostly co-translationally. The energy for this process is provided presumably by the ongoing translation at the ribosome and the pmf. The Sec translocon catalyzes the translocation of certain hydrophilic domains across the membrane and the insertion of hydrophobic transmembrane segments into the lipid bilayer. The translocation of large hydrophilic domains requires energy provided by ATP hydrolysis at SecA, whereas a variety of membrane proteins with shorter, less hydrophilic domains is inserted into the membrane independently of SecA (KUHN, 1988; WERNER et al., 1992; SAAF et al., 1995). The detailed molecular mechanism by which hydrophobic transmembrane segments move out of the aqueous channel to enter the hydrophobic

core region of the phospholipid bilayer still remains largely unresolved. The decision if translocation arrests and hydrophobic segments are released into the lipid bilayer is most likely made in the translocon channel or the lateral gate by interpretation of distinct topological signals (described in the previous chapter 3.2). In general, the efficiency of the insertion process depends on the hydrophobicity and the length of the TM segments as well as the distribution of charged residues (XIE et al., 2007; HESSA et al., 2005 and 2007). Studies of the thermodynamics of membrane insertion by Tara Hessa and colleagues suggest that the insertion is driven by protein-lipid interactions at the translocon-bilayer interface (HESSA et al., 2005). During biogenesis of membrane proteins, the recognition of hydrophobic regions, with sufficient hydrophobicity to serve as stop-transfer signals, leads to an arrest in translocation and to the insertion of the respective TM segment (SAAF et al., 1998b; DUONG & WICKNER, 1998). The putative lateral gate, through which TM segments move out of the channel into the lipid environment of the membrane, is located at the front side of SecY between TM2b/3 and TM7/8 (Figure 1.7B) (VAN DEN BERG et al., 2004; TSUKAZAKI et al., 2008; DU PLESSIS et al., 2009; EGEA & STROUD, 2010; FRAUENFELD et al., 2011; GOGALA et al., 2014; PARK et al., 2014). The transition from a closed to an open state of the lateral gate is most likely the result of a large rotation in the N-terminal half of SecY and resulting movements of SecE and SecG (PARK et al., 2014). Recently obtained cryo-EM structures of a ribosome-SecY complex with an insertion intermediate of nascent proteorhodopsin showed the localization of inserted TM domains outside of the SecY channel in direct vicinity to the potential lateral gate for the first time (BISCHOFF et al., 2014). For efficient insertion into the bilayer, the local lipid environment next to the lateral gate might be influenced by interactions between rRNA helix 59 of the ribosome and phospholipid head groups (FRAUENFELD et al., 2011). Although this is speculative, the local disorder of the bilayer next to the lateral gate may pave the way for the insertion of IMPs.

Regarding polytopic membrane proteins, cross-linking studies suggested that TM segments of polytopic membrane proteins are released through the lateral gate into the bilayer in a sequential mode, one by one or even in pairs (BECK et al., 2001; SADLISH et al., 2005; SKACH, 2009). A predominant role in the Sec-dependent insertion of polytopic membrane proteins was assigned to YidC, which will be discussed in a following chapter.

1.3.4 FOLDING OF α-HELICAL MEMBRANE PROTEINS

Following Anfinsen's dogma, the three-dimensional (3D) structure of a protein is determined by the primary sequence of the polypeptide chain (ANFINSEN, 1973). At least, this is true for small globular proteins. Membrane proteins require another dimension, the specific environment of the lipid bilayer, and most of them have to be inserted into the membrane by protein factors, to acquire their native structures.

1.3.4.1 The two-stage model for membrane protein folding

The widely accepted two-stage model by Popot and Engelmann postulates that folding of membrane proteins occurs in two fundamental stages: insertion and folding (POPOT & ENGELMAN, 1990) (Figure 1.8). In the first stage, individual TM segments are inserted into the bilayer. This process can be both coordinated and driven by a translocon complex or by the membrane insertase YidC. Helix formation is determined by the primary sequence, is driven by the hydrophobic effect and stabilized by hydrogen bonding between the polar groups of the peptide backbone. Thus, the first stage of the model results in the thermodynamic equilibrium of individual TM helices within the lipid bilayer. In the second stage, TM helices interact with each other to form helical bundles. The second stage may include rearrangements and reorientations of TM segments that lead to higher order structures. The two-stage model was later improved to establish a three-stage model. The additional stage describes how helical bundles create a less hydrophobic interior space to incorporate prosthetic groups and additional polypeptides such as coil domains or helices of short length (ENGELMAN et al., 2003). Experimental evidence for this third stage came from folding studies with bacteriorhodopsin protein fragments. Kinetic analysis show that retinal binds after association of the two fragments (POPOT et al., 1987).

1.3.4.2 Early stages of membrane protein folding

The initial formation of helical secondary structures occurs in the ribosome tunnel in a co-translational manner (WOOLHEAD et al., 2004; LU & DEUTSCH, 2005; LIN et al., 2012). Further steps in early folding of membrane proteins are controlled presumably by the translocation and insertion machineries before or after individual transmembrane segments are released into the lipid bilayer.

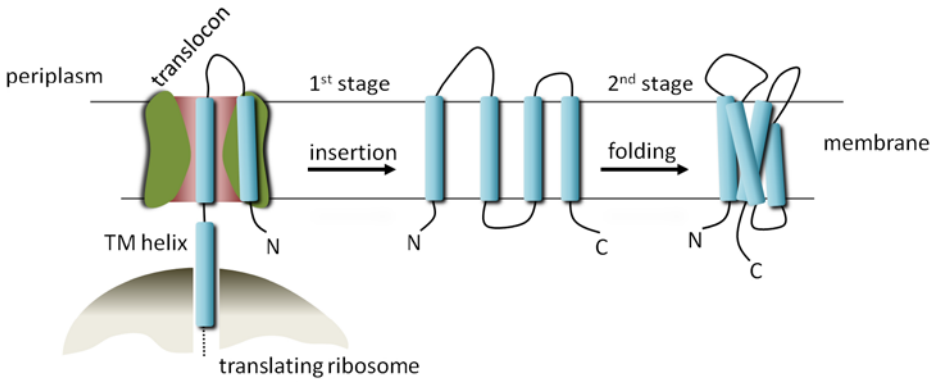

FIGURE 1.8 **Two-stage model for membrane protein folding.** In the first stage, transmembrane segments are inserted into the bilayer. This can be catalyzed by the Sec translocon or YidC (not shown). In the second stage, transmembrane segments interact with each other to form helical bundles.

For the eukaryotic channel forming Sec61α and the bacterial Sec translocon, it has been shown that helices are released sequentially, pair wise or even as a bundle of helices (SKACH, 2009; BECK et al., 2001). For the bacterial Sec translocon early steps in folding of polytopic proteins were proven using a recently developed co-translational *in vivo* assay, in which a pulling force on nascent chains are measured indirectly using a translational arrest peptide (ISMAIL et al., 2012). It has been shown that C-terminal transmembrane segments interact with more N-terminally helices at an early stage when the C-terminal helix portions are released from the Sec channel into the membrane (CYMER & VON HEIJNE, 2013). This suggests that early tertiary interactions occur co-translationally and assign a function for the Sec translocon in early helix packing and/or formation of helical bundles during insertion.

1.3.4.3 Formation of an α-helical bundle

Folding of polytopic α-helical membrane proteins can be considered simply as pairs of interacting helices which form the bulky native structure. High resolution structures of membrane proteins show that helix-helix interactions occur by hydrogen bonds, van der Waals' interactions and salt bridges between neighboring side chains of individual helices. There are several motifs encoded in the primary sequence which determine helix-helix interactions. The best characterized motif is the GXXXG motif (X stands for any AA) in which small glycine residues mediate close approach of helices (LEMMON et

al., 1992). Another motif commonly found in membrane proteins and associated with helix packing is the glycine zipper motif (GXXXGXXXG) (KIM et al., 2005). These motifs are often found in homo-oligomeric channel proteins. For example, the first TM of the pentameric MscL protein in *E. coli* possesses such a glycine zipper which mediates channel formation by helix interactions. In addition, a repeated heptad motif within the sequence leads to the well known 'knobs-into-holes' interaction between helices (LANGOSCH & HERINGA, 1998). Although the presence of polar residues in TM regions is very rare, it has been shown that even single AAs Gln, Glu, Asn and Asp can mediate helix-packing of artificial polyleucine helices (ZHOU et al., 2001).

1.3.4.4 Role of lipids in protein folding

Membrane proteins fold and function in the membrane and there are strong evidences which also support a role of lipids in the folding process. In concert with the Sec translocon, anionic phospholipids assist early folding stages during insertion (VAN KLOMPENBURG et al., 1997; DE VRIJE et al., 1988). Lipids directly involved in folding of membrane proteins are termed 'lipochaperones' as they assist folding like molecular chaperones do. Extensive studies of folding and assembly of LacY suggest that phospholipid PE acts as a lipochaperone (BOGDANOV & DOWHAN, 1999). In addition, the overall lipid composition of the membrane and the thickness, asymmetry and fluidity of the lipid bilayer might influence folding of α-helical membrane proteins (CYMER et al., 2012).

1.4 THE MEMBRANE INSERTASE YidC

The membrane bound YidC protein is presumably the simplest translocon for biogenesis of inner membrane proteins in *E. coli*. While the vast majority of inner membrane proteins are inserted via the Sec translocon, a not negligible number of membrane proteins use YidC to achieve membrane insertion. For the first time, this has been shown by Samuelson et al. in 2000, when they uncovered the central role of YidC for insertion of the small phage protein M13 procoat (SAMUELSON et al., 2000). In addition, in the same year YidC has been shown to be a component of the Sec translocon and that it can be cross-linked to Sec-dependent membrane proteins (SCOTTI et al., 2000).

Since then, when these first exciting evidences for a new membrane insertion pathway have been found, a lot of work has been done to draw a more and more precise picture of YidC mediated membrane insertion and the cooperation of YidC with the Sec translocon. YidC seems to work additionally as a membrane bound chaperone and to assist in folding of inner membrane proteins. Furthermore, it functions in supporting the assembly of a variety of oligomeric membrane protein complexes (DALBEY et al., 2014).

1.4.1 YidC/Oxa1/Alb3 PROTEIN FAMILY AND FUNCTIONAL CONSERVATION AMONG HOMOLOGS

E. coli YidC is a member of the YidC/Oxa1/Alb3 protein family that is widely spread in all domains of life (Figure 1.9). Members of the protein family have been identified in overall genome analyses upon the discovery, that Oxa1 (oxidase assembly 1) protein in yeast mitochondria is involved in biogenesis of cytochrome oxidase (BONNEFOY et al., 1994; BAUER et al., 1994). Bacteria, eukaryotes and archaea (i. e. Euryarchaeota) comprise at least one evolutionary conserved YidC protein involved in the biogenesis of membrane proteins in the cytoplasmic membrane of bacteria and archaea, the inner membrane of mitochondria (Oxa proteins) and the thylakoid membrane of chloroplasts (Alb proteins), respectively (LUIRINK et al., 2001; YEN et al., 2001; ZHANG et al., 2009; FUNES et al., 2009; FUNES et al., 2011). The conserved catalytic core region of the homologous proteins is represented by the five C-terminal TM domains (YEN et al., 2001) (Figure 1.9). The highest sequence identity among bacterial and eukaryotic homologs has been found in TM2 and TM3 and the overall sequence identity of YidC to Oxa1 (from *Saccharomyces. cerevisiae*) and to Alb3 (from *Arabidopsis thaliana*) amounts moderate 20 % and 30 %, respectively (KIEFER & KUHN, 2007). In contrast to *E. coli* YidC, the eukaryotic homologs Oxa1, Alb3, Alb4, as well as YidC2 of Gram-positive bacteria contain extended positively charged C-terminal sequences of variable length with a predicted coiled-coil structure, which is proposed to bind RNCs and ribosomes during co-translational insertion.

The function of YidC-like proteins is conserved among the family members. They play a central role in the biogenesis of some subunits of large energy-transducing complexes. Nevertheless, it is surprising that the YidC-like proteins are exchangeable among different species and domains. For example, respiratory defects in *S. cerevisiae* caused by

the depletion of *oxa1,* can be rescued by *E. coli* YidC (PREUSS et al., 2005) and vice versa, *A. thaliana* Alb3 (JIANG et al., 2002) and *S. cerevisiae* Oxa1 (VAN BLOOIS et al., 2005) can complement the growth defect of a YidC depletion strain. In bacteria, YidC homologs of Gram-positives can complement for depleted *yidC* in Gram-negative *E. coli* (DONG et al., 2008; SALLER et al., 2009).

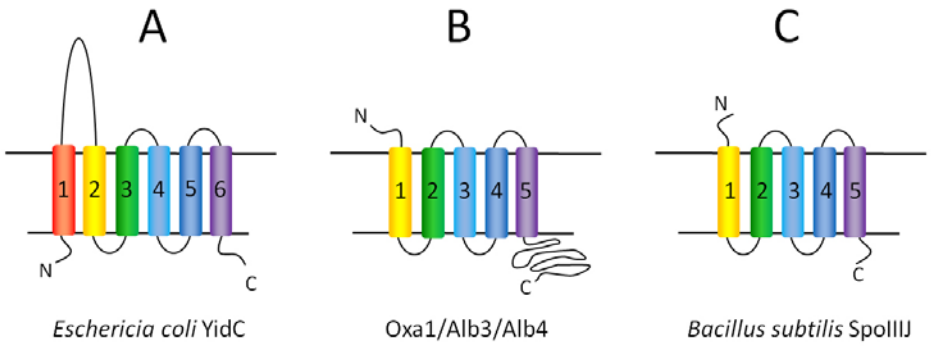

FIGURE 1.9 **Membrane topology of YidC proteins from the YidC/Oxa1/Alb3 protein family.** The evolutionary conserved core domain of the homolog proteins comprises the TM domains (TM2-6 for YidC from *E. coli* and TM1-5 for homolog proteins). Homolog TM domains are represented in the same color. **(A)** Topology of *E. coli* YidC based on experimental data from SAAF et al., 1998a. *E. coli* YidC possesses an additional N-terminal transmembrane segment (in red). **(B)** Oxa1 from mitochondria and Alb3/Alb4 from thylakoid membranes. **(C)** SpoIIIJ (YidC1) from *Bacillus subtilis*. YidC2 of Gram-positive bacteria possesses a long C-terminal domain as has been found in the Oxa1/Alb3/Alb4 homologs.

1.4.1.1 Oxa proteins in the inner membrane of mitochondria

In mitochondria, two isoforms of Oxa proteins, Oxa1 and Oxa2, are located in the inner membrane. Both facilitate the insertion of mitochondrially-encoded integral membrane proteins. Oxa1 has been shown to promote insertion of various subunits of the cytochrome c oxidase (Cox proteins) (HE & FOX, 1997; HELL et al., 1997; HELL et al., 2001) and to assist assembly of both cytochrome c oxidase and ATP synthase complex (ALTAMURA et al., 1996; BONNEFOY et al., 1994; BAUER et al., 1994). Oxa1 mediates insertion of mitochondrially-encoded proteins co-translationally (HELL et al., 2001; JIA et al., 2003) and on the other hand directly binds its substrates in a post-translational way, as it has been shown for subunit 9 of ATP synthase (JIA et al., 2007). For the insertion of the polytopic multidrug transporter Mdl1 protein it has been previously shown, that Oxa1 cooperates with the translocase of the inner membrane (TIM23) (BOHNERT et al., 2010). Reconstitution of Oxa1 into a lipid bilayer and subsequent biophysical analysis suggests that Oxa1 forms a voltage-gated, hydrophilic channel which can be activated by

the binding of small peptides of natural substrate proteins (KRÜGER et al., 2012). The second isoform, Oxa2 (also called Cox) was initially identified in a screen of mutants impaired in activity of the cytochrome c oxidase (TZAGOLOFF et al., 1990). Oxa2/Cox is required for the post-translational insertion of the C-terminal part of Cox2p (SARACCO & FOX, 2002). Here, Oxa2 cooperates with Oxa1, which promotes insertion of the N-terminal region of Cox2p (HE & FOX, 1997; HELL et al., 1997).

1.4.1.2 Alb proteins in the thylakoid membrane of chloroplasts

The thylakoid-localized membrane protein Alb3 was designated to be the homolog of yeast mitochondria Oxa1 with a similar, essential insertase activity in chloroplasts thylakoid membranes (SUNDBERG et al., 1997). So far, the only substrates, destined for the Alb3 mediated insertion pathway, are nuclear-encoded light-harvesting chlorophyll-binding proteins (LHCPs) of the photosystems I and II (MOORE et al., 2000). To exert membrane biogenesis of LHCPs, Alb3 cooperates in a post-translational way with chaperones of the stroma, the SRP system of chloroplasts (cpSRP43, cpSRP54), and its receptor FtsY. Furthermore, Alb3 cooperates most likely with the cpSec translocase after import of LHCPs into the chloroplast stroma has occurred (reviewed in ANKELE et al., 2012). Another, less important isoform found in thylakoid membranes, is Alb4. Alb4 exhibits a less pronounced phenotype than the Alb3 albino phenotype does (GERDES et al., 2006; BENZ et al., 2009). Unlike Alb3, Alb4 does not function as an insertase but rather acts as a stabilization and assembly factor of chloroplast ATP synthase (BENZ et al., 2009).

1.4.2 X-RAY STRUCTURE OF YidC2 FROM BACILLUS HALODURANS

Like many other Gram-positive bacteria, the genomes of *Bacillus* species contain two *yidc* genes, encoding for the membrane proteins YidC1 (SpoIIIJ) and YidC2 (YqjG) (FUNES et al., 2009). Knock-out studies have shown, that at least one isoform is sufficient for cell viability, whereas the inactivation of both genes is lethal (MURAKAMI et al., 2002). Both isoforms are synthesized with an N-terminal cleavable signal sequence and contain the conserved core-region of five TM domains, whereas only YidC2 comprises an extended C-terminal tail like Oxa1 and Alb3 (TJALSMA et al., 2003; FUNES et al., 2009).

The recently obtained high resolution x-ray structure of *B. halodurans* YidC2 at 2.4 Å, obtained by KUMAZAKI et al., constitutes a major breakthrough in the field of YidC mediated membrane insertion. The structure reveals a hydrophilic groove, formed by the five TM domains, which is open towards both the hydrophobic core of the lipid bilayer and the cytoplasm, but closed at the periplasmic side by tightly packed hydrophobic side chains of the TM helices and the connecting periplasmic loops (KUMAZAKI et al., 2014a).

KUMAZAKI et al. presented the nearly identical structures of two YidC2 constructs (PDB entries: $3WO6_{(residues\ 27\text{-}266)}$ and $3WO7_{(residues\ 27\text{-}267)}$). Both are lacking the N-terminal signal sequence and most of the C-terminal sequences. In the following I will refer to the structural details of $YidC2_{(residues\ 27\text{-}266)}$ as represented in Figure 1.10. The obtained structure shows the detailed organization of the periplasmic domains, the TM helices of the core region (TM1-5) and the cytoplasmic domains. The P1 domain is shown as an amphipathic helix lying nearly in parallel to the membrane interface which is consistent with the x-ray structure of the corresponding region in *E. coli* YidC (OLIVER & PAETZEL, 2008; RAVAUD et al., 2008). The TM1 helix is relatively long, kinked at conserved proline residues (P74, P98) and protrudes into the C1 domain, consisting of two α-helices which form a helical hairpin of mostly hydrophilic residues at the cytoplasmic surface of the membrane. The cytoplasmic halves of the core domain TM1-5 form the hydrophilic groove which is coated by conserved hydrophilic residues, distributed in all five TM domains. Interestingly, a highly conserved arginine residue in TM1 (R72) is located in the central region of the hydrophilic cavity (shown as sticks and labeled in Figure 1.10A and C).

The hydrophobic core of YidC2 consists of the tightly packed periplasmic halves of TM1-5 and the P2 domain, both capping the hydrophilic cavity towards the periplasm (Figure 1.10 C). The hydrophobic gasket has been shown to be impermeable for ions and water molecules (KUMAZAKI et al., 2014a). However, this finding contradicts recently obtained experimental data on the reconstituted YidC homolog Oxa1 from yeast mitochondria, suggesting the formation of a voltage-gated hydrophilic channel pore which is permeable for cations (KRÜGER et al., 2012).

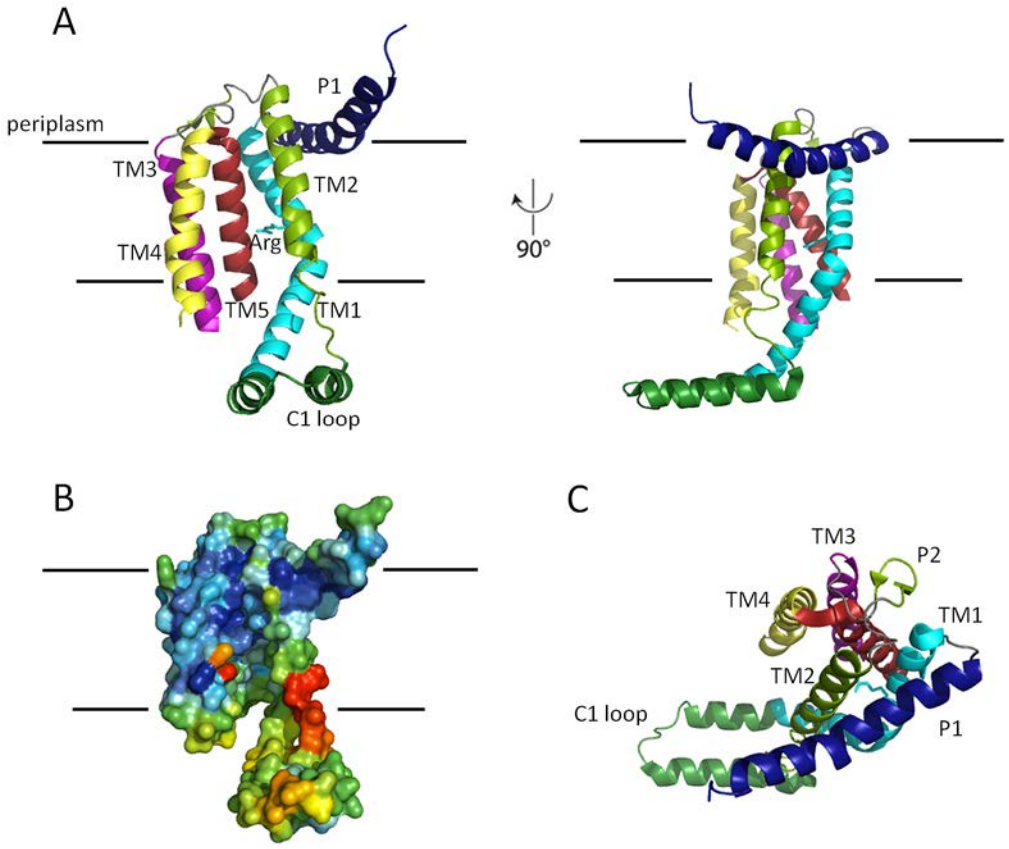

FIGURE 1.10 **Structure of *Bacillus halodurans* YidC2 (PDB entry 3WO6). (A)** Cartoon representation viewed in the plane of the membrane, looking into the hydrophilic groove (left). TM domains and connecting loops are numbered. The conserved Arg residue R72 is labeled and shown as sticks. **(B)** Surface representation orientated as shown in (A), looking directly into the groove. The crystallographic B-factor of the overall structure which reflects mobility and flexibility of structural elements is rainbow colored from red (highest B-factor – reflecting highest mobility and flexibility) to blue (lowest B-factor). **(C)** View from the periplasm, showing that the hydrophilic groove is closed towards the periplasm. (Adapted from

The local crystallographic B-factor (Debye-Waller factor) indicates static and dynamic disorder of atoms in the protein crystal. Together with molecular dynamics simulations the overall B-factor assign the highest flexibility of YidC2 to the C1 region and the cytoplasmic halves of the TM regions. However, the hydrophobic core shows a lower B-factor and seems to be the rigid part of the YidC2 protein (Figure 1.10B). In addition, a superimposition of the two obtained structures, $YidC_{(residues\ 27\text{-}266)}$ and $YidC_{(residues\ 27\text{-}267)}$ shows a structural flexibility of the hydrophilic groove and the C1 region (KUMAZAKI et al., 2014a).

Based on the solved crystal structure, KUMAZAKI et al. used site directed mutagenesis of the *B. subtilis* orthologue SpoIIIJ for the determination of functionally important regions. First of all, single point mutations of the conserved charged R73 residue in TM1

(corresponding to R72 in YidC2) to M, A, Q, E or D significantly decreased the insertion activity of SpoIIIJ and resulted in a lethal phenotype as observed in a complementation assay. Solely the substitution of R73 by a positively charged lysine can partially rescue the insertion of MifM, which has been used in this assay as a model substrate of SpoIIIJ (CHIBA et al., 2009). Other single residues of the hydrophilic groove do not seem to be as important for activity, since ten of the polar residues coating the groove can be replaced by the non-polar amino acid alanine. KUMAZAKI et al. then investigated the importance of the amphipathic helix P1, the groove-capping P2 domain and the highly flexible C1 region. The results show clearly that the partial deletion of distinct parts of the tested domains impaired both the insertion of MifM and cell viability. This is mostly consistent with data from experiments assessing the functional important regions of *E. coli* YidC (JIANG et al., 2003).

In summary, the first high resolution structure of a YidC-like protein remarks a milestone in discovering the molecular organization and function of YidC family proteins. The structure reveals a remarkable tertiary arrangement of a protein inside the membrane, since the functional important hydrophilic groove is open towards the lipid bilayer and the cytoplasm but closed to the periplasm (KUMAZAKI et al., 2014a).

1.4.3 THE MEMBRANE INSERTASE YidC FROM ESCHERICHIA COLI

The membrane insertase YidC from *E. coli* is a 61 kDa protein of 548 AAs embedded in the cytoplasmic membrane (Figure 1.11A). YidC itself requires SRP/FtsY and SecYEG/SecA for proper targeting and membrane insertion. It is assumed, that approximately 2500 to 3000 copies are located in the inner membrane of *E. coli* cells during mid-exponential phase of growth (URBANUS et al., 2002).

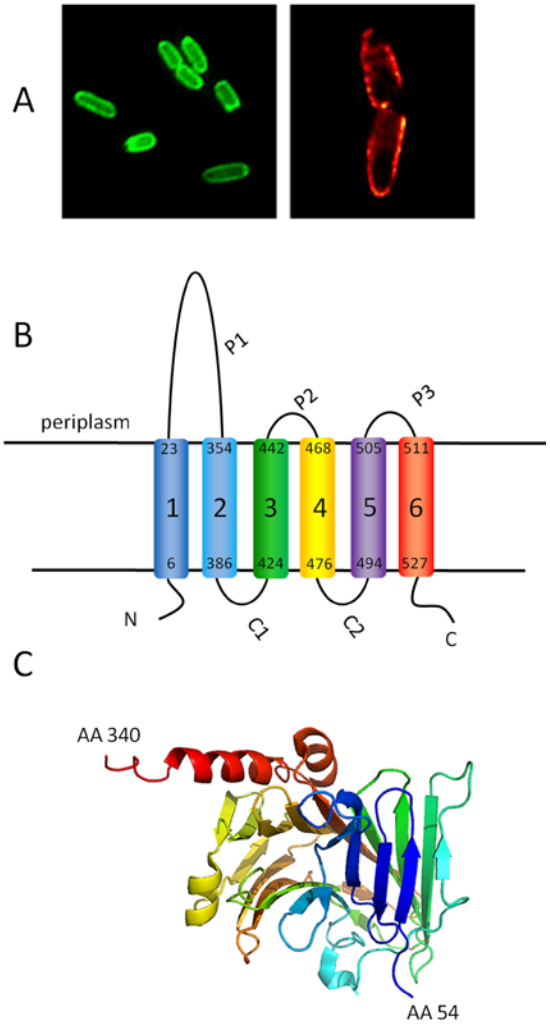

FIGURE 1.11 **The membrane insertase YidC from *E. coli*: Membrane localization, topology map and crystal structure of the periplasmic loop P1. (A)** Membrane localization of Citrine:YidC fusion protein expressed in *E. coli* cells. Citrine:YidC was expressed using the endogenous *yidC* promotor region upstream of the open reading frame to monitor YidC localization in as natural conditions as possible. *E. coli* cells expressing Citrine:YidC were cultured until having reached mid-exponential phase of growth. Wide-field fluorescence image (left) and STED microscopy image of dividing *E. coli* cells at a resolution of 50 nm (right). **(B)** Topology map of *E. coli* YidC based on experimental data from SAAF et al., 1998a and data from the crystal structure solved by KUMAZAKI et al., 2014b. TM segments are colored and the predicted length of TM segments is indicated with flanking residues numbers. Hydrophilic loops on the periplasmic (P) and cytoplasmic (C) side of the membrane are numbered consecutively. **(C)** Crystal structure of periplasmic loop P1 from OLIVER & PAETZEL, 2008 (PDB entry 3BLC). The structure is rainbow colored from the N-terminus (blue) to the C-terminus (red).

Although a local accumulation of YidC proteins at the cell poles was proposed (URBANUS et al., 2002), data obtained from high resolution fluorescence microscopy suggest that YidC is rather distributed in the whole cell (KLENNER, GERKEN & KUHN, unpublished data) (Figure 1.11A). Hydrophobicity and PhoA fusion analyses suggest a membrane topology of six TM domains with an N_{inside}-C_{inside} orientation (Figure 1.11B) (SÄÄF et al., 1998a). The N-terminal TM domain serves as an uncleaved signal anchor sequence and is not present in other members of the protein family (SÄÄF et al., 1998a). TM1 and TM2 are connected by a large hydrophilic domain, called loop P1, of approximately 320 AAs. Until last, structural information of *E. coli* YidC was limited to high resolution structures from the loop P1, and a low resolution projection map of reconstituted YidC from cryo-EM analysis of 2D crystals (OLIVER & PAETZEL, 2008; RAVAUD et al., 2008; LOTZ et al., 2008). Recently, the crystal structure of *E. coli* YidC was refined at 3.2 Å resolution (KUMAZAKI et al., 2014b). The characteristics of the structure will be described in detail in the following chapter 1.4.3.1.

The loop P1 of *E. coli* YidC consists primarily of a large β-supersandwich with a short α-helical domain within and at the edge of the sandwich, respectively, and two α-helices at the very C-terminus, which seem to be a flexible linker between the P1 loop and the downstream core domain (OLIVER & PAETZEL, 2008; RAVAUD et al., 2008; KUMAZAKI et al., 2014b) (Figure 1.11C). Surprisingly, approximately 92 % of the large periplasmic domain P1 can be deleted without affecting YidC activity (JIANG et al., 2003). However, it has been found that regions of the loop P1 mediate interaction with SecF (XIE et al., 2006). Recently obtained data from cross-linking studies by HENNON & DALBEY gathered first insights into the orientation of the C-terminal transmembrane domains TM2-6 constituting the core region of YidC. Their results suggest that TM3, TM4, TM5 and TM6 each have a helical face towards TM2 (HENNON & DALBEY, 2014). In addition, the cytoplasmic regions of TM3-TM6 seem to be very flexible, based on the stable intermolecular cross-links between TM2 and a range of residues in TM3-TM6, that have been observed with bifunctional cross-linkers of different spanner length (HENNON & DALBEY, 2014). The spatial arrangement of the TM helices as well as the flexibility of the cytoplasmic halves of the TM helices obtained by HENNON & DALBEY seem to be partially consistent with the recently obtained crystal structure of *B. halodurans* YidC2 and *E. coli* YidC (KUMAZAKI et al., 2014a; KUMAZAKI et al., 2014b). The C-terminal end of *E. coli* YidC

(16 AAs) is short and moderately positively charged compared to the corresponding regions in various homologs from other species (KIEFER & KUHN, 2007; SEITL et al., 2013). In the past, it has been discussed controversially, whether YidC acts as a monomer or in an oligomeric state. Evidence that YidC is mostly present in a dimeric form has come from blue native polyacrylamide gel electrophoresis (BN-PAGE) experiments (BOY & KOCH, 2009). In addition, the placement of a single cysteine residue in TM1 converts overexpressed YidC almost completely into the dimeric form (KLENNER, unpublished data). A 2D projection map of reconstituted, detergent-solubilized *E. coli* YidC suggests a dimeric state with an anti-parallel face-to-face orientation (LOTZ et al., 2008). The low resolution cryo-EM structures of both a RNC-YidC and RNC-Oxa1 complex have drawn a similar picture: Here, YidC and Oxa1 were modeled as dimers bound to the translating ribosome (KOHLER et al., 2009). Oxa1 dimeric and tetrameric state has been suggested to be the active unit (NARGANG et al., 2002; KRÜGER et al., 2012). SpoIIIJ from *B. subtilis* is able to form dimers presumably stabilized by a disulfide bound between a conserved cysteine in TM2 of each monomer (CÔRTE et al., 2014). Interestingly, sporulation is blocked when the cysteine is mutated, thus the active state of SpoIIIJ seems to be the dimeric form. On the other hand, recent studies propose a monomeric state of *E. coli* YidC to be the functional unit. First of all, the crystal structure of YidC2 and *E. coli* YidC clearly shows YidC as a monomer (KUMAZAKI et al., 2014a; KUMAZAKI et al., 2014b). In addition, two cryo-EM structures have shown a single copy of YidC bound to an active ribosome (SEITL et al., 2013; WICKLES et al., 2014). Furthermore, fluorescence cross-correlation spectroscopy (FCCS) studies revealed that a single copy of YidC is sufficient to bind an active ribosome (KEDROV et al., 2013). Examination of the overexpressed and purified SecYEG-SecDF-YajC-YidC holocomplex by BN-PAGE shows, that YidC is present within the complex as a monomer (SCHULZE et al., 2014).

Taken together, YidC-like proteins seemingly tend to dimerize and oligomerize. The physiological relevance of this observation is not clear in all cases. It has been shown that *E. coli* YidC is fully active in a monomeric state.

1.4.3.1 Crystal structure of Escherichia coli YidC

Recently, KUMAZAKI et al. have published the structure of *E. coli* YidC at 3.2 Å resolution (KUMAZAKI et al., 2014b). The structure was determined by using structural data of the P1 domain (PDB entry 3BS6; RAVAUD et al., 2008) and the TM regions of *B.*

halodurans YidC2 (PDB entry 3WO6; KUMAZAKI et al., 2014a) for molecular replacement. The structure comprises the TM domains 2-6 and the P1 and C1 regions (Figure 1.12 and Figure 1.13). The first TM domain (TM1) as well as the C2, C3 and the tail regions is structurally disordered.

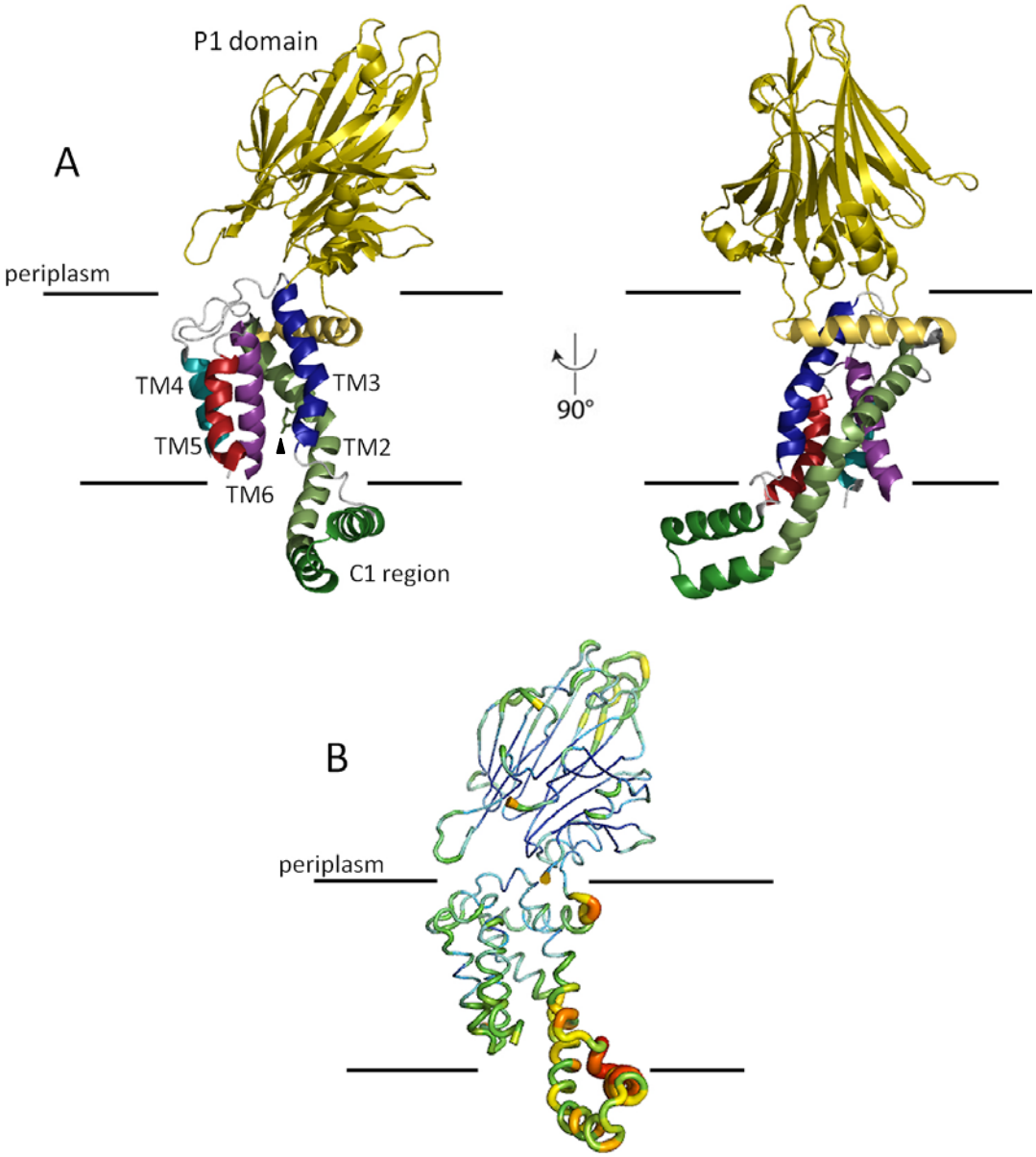

FIGURE 1.12 **Crystal structure of *Escherichia coli* YidC (PDB entry 3WVF). (A)** Cartoon representation viewed in the plane of the membrane, looking into the hydrophilic groove (left) and viewed from the side (right). The conserved Arg residue R366 in the center of the hydrophilic groove is shown as sticks and indicated with a black arrowhead (left). **(B)** Representation of local B-factors orientated as shown in (A), looking directly into the groove. The crystallographic B-factor of the overall structure which reflects mobility and flexibility of structural elements is rainbow colored from red in thick lines (highest B-factor – reflecting highest mobility and flexibility) to blue in thin lines (lowest B-factor).

The arrangement of the five TM domains is similar to that of *B. halodurans*: a hydrophilic groove is formed by loosely packed portions of TM α-helices 1-5 at the cytoplasmic leaflet and tightly packed domains of the α-helices in the periplasmic leaflet, capping the groove towards the periplasm. The groove is coated by conserved hydrophilic residues distributed in TM domains 2-6 and opened towards the cytoplasmic side and the membrane interior by a gap between TM3 and TM5 (KUMAZAKI et al., 2014b) (Figure 1.12) . In the central region of the hydrophilic cavity, a highly conserved residue of TM2, i.e. R366, is located at exactly the same position as the corresponding residue R72 in the structure of *B. halodurans* YidC2 (Figure 1.10 and Figure 1.12).

In the *E. coli* YidC structure the large P1 domain is identical to the previously published structures of the P1 domains (OLIVER & PAETZEL, 2008; RAVAUD et al., 2008; KUMAZAKI et al., 2014b). The extracellular P1 domain is linked to TM2 via a short membrane embedded α-helix (Figure 1.12A). Residues of the P1 domain forms several hydrogen bonds with residues of the P2 domain (connecting TM3 and TM4) and TM3, suggesting that the spatial orientation of the P1 domain is stabilized by these means and in addition by interactions with membrane lipids (KUMAZAKI et al., 2014b; RAVAUD et al., 2008).

It is consistent with the *B. halodurans* structure that the most flexible region of *E. coli* YidC was assigned to the C1 region, consisting of two antiparallel α-helices at the cytoplasmic leaflet of the membrane and to the N-terminal region of TM3(Figure 1.12B). This suggests that the flexibility of the C1 domain and TM3 is a conserved structural feature which is important for YidC function.

In summary, the first crystal structure of *E. coli* YidC reveals that the formation of a hydrophilic groove by the transmembrane core domains TM2-6 is the structural basis for YidC-mediated insertion. The sequence alignment of the YidC/Oxa1/Alb3 family members shows clearly a high conservation of the positively charged residue which is located in the center of the hydrophilic groove (Figure 1.13).

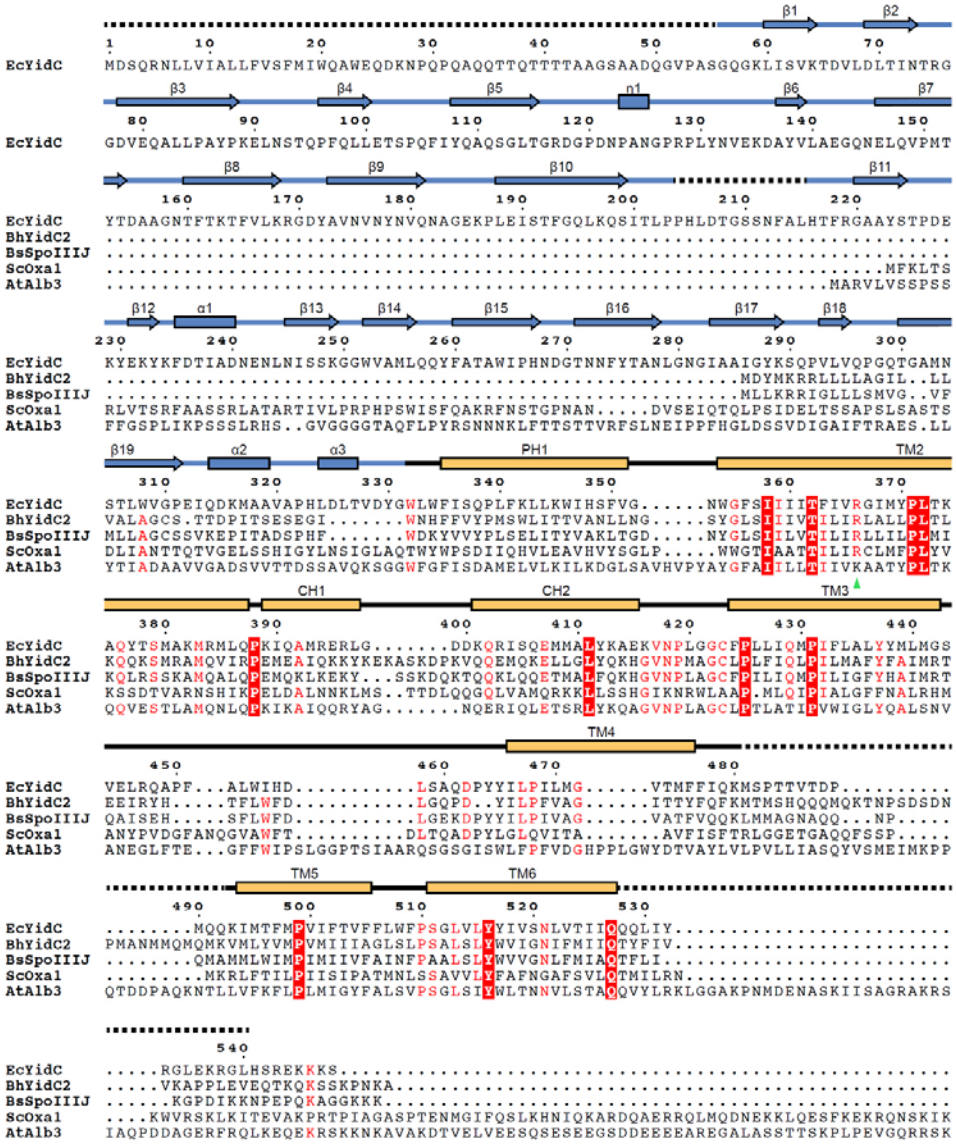

FIGURE 1.13 **Sequence alignment of representatives from the YidC/Oxa1/Alb3 family and secondary structure information of *E. coli* YidC based on the crystal structure.** Sequence homology between *Escherichia coli* (EcYidC), *Bacillus halodurans* YidC2, (BhYidC2), *Bacillus subtilis* SpoIIIJ (BsSpoIIIJ), *Saccharomyces cerevisiae* Oxa1 (ScOxa1) and *Arabidopsis thaliana* Alb3 (AtAlb3). The most conserved residues are highlighted in red boxes and highly conserved residues are written in red. The green triangle indicates the highly conserved positively charged residue which is located in the center of the hydrophilic groove of the solved *B. halodurans* and *E. coli* crystal structure. Numbers above the alignment refer to the amino acid sequence of *E. coli* YidC. The secondary structure of *E. coli* YidC is indicated by arrows (for β-strands) and cylinders (for α-helices). Structural features of the P1 domain and the transmembrane core region are highlighted in blue and orange, respectively. Dashed lines indicate the structurally disordered regions. Adapted from KUMAZAKI et al., 2014b.

1.4.3.2 YidC and the ribosome

The long positively charged C-terminus of many YidC homologs was assigned to enable an electrostatic interaction with ribosomes during co-translational insertion (JIA et al., 2003; PREUSS et al., 2005; KOHLER et al., 2009; GRUSCHKE et al., 2010; HAQUE et al., 2010; PALMER et al., 2012; SEITL et al., 2013). The C-terminal end of YidC is short (approximately 18 AAs) compared to the homologs (approximately 100) and less positively charged (+7 compared to ±20) (SEITL et al., 2014). An efficient association of *E. coli* YidC with ribosomes has been shown by using YidC with a C-terminal hexa-histidine tag (protonated at acidic pH) (KOHLER et al., 2009) and a chimera YidC with the fused C-terminus of planctomycete *Rhodopirellula baltica* (SEITL et al., 2013; WICKLES et al., 2014). A recent study revealed, that the binding of detergent–solubilized histidine tagged YidC to the ribosomes is significantly decreased under physiological conditions, when histidine residues are not protonated (KEDROV et al., 2013). However, the reconstitution of YidC into lipid-nanodiscs leads to an efficient ribosome binding at the physiological condition at pH 7.4. Interestingly, the interaction is increased, when the ribosome is loaded with an emerging nascent chain of a substrate protein (KEDROV et al., 2013). Although YidC seems to bind not only to the exit tunnel (WELTE et al., 2012), ribosome binding sites for YidC were designated to the ribosomal proteins L23, L24, L29 and to RNA helix 59, located in the vicinity of the ribosomal tunnel exit (KOHLER et al., 2009; SEITL et al., 2013; WICKLES et al., 2014).

1.4.3.3 Functionally important regions of Escherichia coli YidC

Functionally critical regions of *E. coli* YidC have been defined in a detailed study using mutagenesis and subsequent complementation and insertion assays (JIANG et al., 2003). The results, summarized in Figure 1.14, indicate that the C-terminal residues of loop P1, in the crystal structure seen as an amphipathic helix, and the hydrophilic loops P2 and P3 are functional domains. In addition, JIANG et al. found that the core region of YidC, comprising TM2-6, is critical for YidC activity. However, TM4 and TM5 can be replaced by non-homologous TM domains of leader peptidase without loss of function. In TM2, TM3 and TM6 single point mutations of I361, L436 and Y516 to the residue serine lead to inactivation of YidC. Recently, reinvestigation of the importance of the C1 domain has shown that deletion of the entire C1 domain (residues 371 to 416) leads to a lethal phenotype, when the expression of the chromosomal encoded *yidC* gene was inhibited (CHEN et al., 2014).

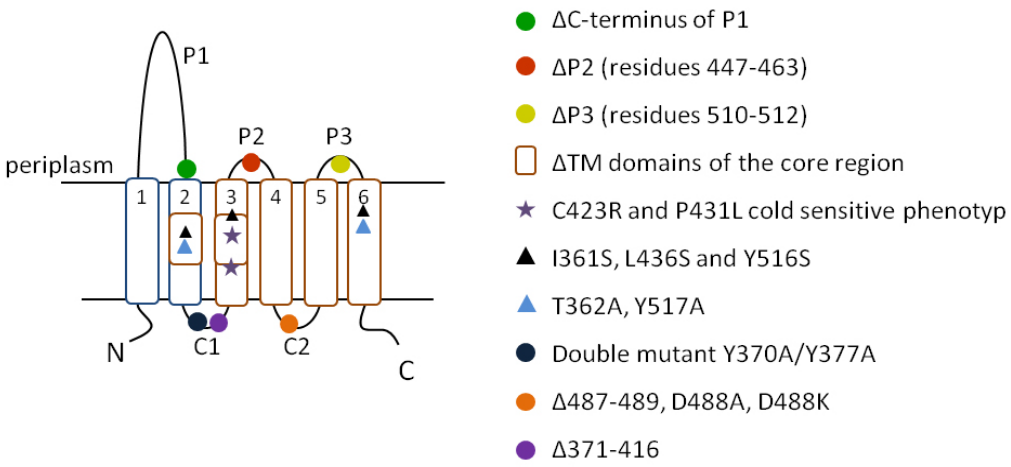

FIGURE 1.14 **Functionally important regions of *E. coli* YidC.** Several deletions or even single point mutations in distinct domains lead to a loss of YidC activity (indicated by symbols in the topology model of YidC on the left and described in the legend on the right). The illustration summarizes results from JIANG et al. 2003, YUAN et al. 2007, WICKLES et al. 2014 and CHEN et al., 2014.

Another study has shown, that single point mutations of two residues located in TM3 lead to a cold-sensitive phenotype (P431L, C423R; Figure 1.14) (YUAN et al., 2007). In addition, a suppressor mutation of C423R, to rescue the lethal phenotype at the nonpermissive temperature, has been found in TM2 at residue T362 when mutated to glutamic acid (T362E). Further evidence for functionally important residues has been adduced recently by WICKLES et al. Based on their structural model of *E. coli* YidC, two single residues which are suggested to be important for strong helix-helix interactions to stabilize the core helix bundle were mutated. Consequently, the mutation of T362 (TM2) and Y517 (TM6) to the non-polar residue alanine completely inactivated YidC (WICKLES et al., 2014). In addition, deletion or mutation of residues identified to bind translating ribosomes leads to a lethal phenotype, i. e. Δ487-489 and D488K/A (C2 loop) and the double mutation Y370A/ Y377A (C1 loop).

Based on the recently obtained crystal structure of *B. halodurans* YidC2, mutational analysis in *B. subilis* SpoIIIJ revealed a functionally critical role for the conserved arginine residue (R72) located in the center of the hydrophilic groove (KUMAZAKI et al., 2014a). In *E. coli* YidC, the serial mutation of five residues in the corresponding region to serine (R366-GIM-Y370 in TM2) did not have an effect on the insertion of a model substrate (JIANG et al., 2003) but the single point mutations R366E and R366D

inactivated YidC, since the insertion of Pf3-Lep was blocked (CHEN et al., 2014). Further growth complementation assays with *E. coli* YidC variants with changes at residue 366 showed, that the conserved R366 residue is not that strictly required for the function of *E. coli* YidC as found for *B. halodurans* YidC2 (KUMAZAKI et al., 2014a; KUMAZAKI et al., 2014b).

1.4.3.4 Cellular response upon YidC depletion

Quantitative analyses of both changes in gene expression and the constitution of the inner membrane proteome upon YidC depletion provide a detailed insight into the reaction of cells upon the depletion of YidC and underline the physiological importance of YidC for essential cellular processes (PRICE et al., 2010; WANG et al., 2010; WICKSTRÖM et al., 2011b). YidC depletion results in growth defects after 5 hours (h) - YidC depleted cells are significantly increased in length compared to wild type cells. Most likely, this is a result of the loss of membrane integrity upon YidC depletion. Additionally, the depletion leads to a significantly decreased motility (WANG et al., 2010). A typical effect of YidC depletion is the overexpression of the phage shock protein A (PspA), which is thought to be induced by conditions affecting the pmf to maintain integrity of the cytoplasmic membrane (VAN DER LAAN et al., 2003; JONES et al., 2003; DARWIN et al., 2005). In case of YidC depletion, defects in the assembly of cytochrome *o* oxidase and the F_oF_1-ATP synthase lead to a reduction of the pmf (VAN DER LAAN et al., 2003). Interestingly, transcriptome studies revealed that the level of *pspA* transcripts is 15–fold higher in YidC depleted cells, compared to the induction caused by disrupting the pmf with the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) (WANG et al., 2010). This suggests that the strong PspA response is not only coupled to the disruption of the pmf. The activation of the Cpx stress pathway, which is generally induced after alteration of inner membrane integrity (reviewed in RAIVIO, 2014) has also been observed in YidC depleted cells (WANG et al., 2010; SHIMOHATA et al., 2007). Another effect observed upon YidC depletion is the upregulation of cytoplasmic chaperones, like DnaK, DnaJ, GroEL, and ClpB (PRICE et al., 2010). Analysis of the inner membrane proteome using two-dimensional blue native/SDS-PAGE (2D BN/SDS-PAGE) has shown that - in addition to DnaK and GroEL - the expression of periplasmic and membrane embedded chaperones like PpiD (induced by the Cpx stress pathway), OppA, FtsH, HflC, Hflk is highly upregulated in YidC depleted cells (WICKSTRÖM et al., 2011b).

1.4.4 YidC MEDIATES MEMBRANE PROTEIN INSERTION AND ASSISTS IN FOLDING AND ASSEMBLY

So far, the number of proteins destined to use solely YidC for membrane integration is relatively limited (Figure 1.15). The function of YidC as a Sec-independent working membrane insertase has been initially shown for the insertion of small phage coat proteins M13 procoat and Pf3 coat, both of which are sec-independent proteins (WOLFE et al., 1985; ROHRER & KUHN, 1990). M13 procoat consists of 79 residues and comprises one TM domain and an N-terminal cleavable signal sequence. Pf3 coat is a protein of 44 residues and consists of one TM domain. It has been shown that membrane insertion of both proteins was highly impaired in cells depleted of YidC (SAMUELSON et al., 2000; SAMUELSON et al., 2001; CHEN et al., 2002). That the observed insertion deficiency was not substantiated by a pleiotropic effect as a result of YidC depletion has been shown for both phage proteins by the observation of an efficient insertion into proteoliposomes containing YidC as the unique proteinous factor (SEREK et al., 2004; STIEGLER et al., 2011).

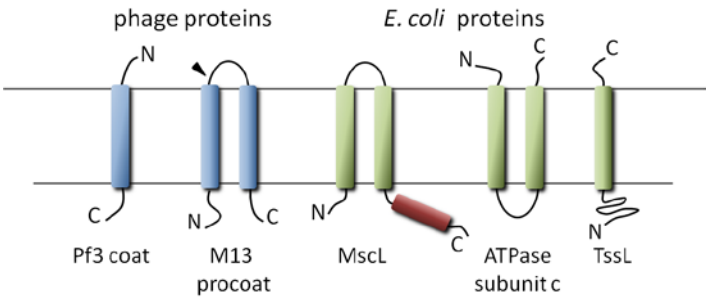

FIGURE 1.15 **YidC substrates and their membrane topology.** YidC mediates the insertion of small phage coat proteins Pf3 coat and M13 procoat (left side, in blue). M13 procoat is synthesized with an N-terminal cleavable signal sequence (processing by leader peptidase after insertion is indicated with an arrowhead). In addition, YidC mediates the insertion of endogenous *E. coli* proteins MscL, possessing a cytoplasmic α-helical domain (in red), subunit c of the ATPase (F_oc) and the TAMP protein TssL, a component of the Type IV secretion system of enteroaggregative *E. coli* (right side, in green).

In addition, a contact of inserting Pf3 coat protein to YidC has been demonstrated in extensive studies (CHEN et al., 2002; YU et al., 2008; GERKEN et al., 2008; WINTERFELD et al., 2009; IMHOF et al., 2011; ERNST et al., 2011; WINTERFELD et al., 2013).

The first endogenous *E. coli* substrate has been identified to be the subunit c of the F_1F_o-ATP synthase (F_oc), a protein consisting of 79 residues and two TM domains connected by a cytoplasmic loop (Figure 1.15). Membrane-inserted F_oc protein oligomerizes to a decamer forming the proton-conducting c-ring of the membrane embedded F_o part of the ATP synthase. Similar to the phage proteins, membrane insertion of F_oc was decreased in cells depleted of YidC (VAN DER LAAN et al., 2003). Later it has been shown in detail that the insertion of F_oc strictly depends on YidC (YI et al., 2003; VAN BLOOIS et al., 2004; VAN DER LAAN et al., 2004). The second endogenous YidC substrate is presumably the mechanosensitive channel protein MscL (FACEY et al., 2007; PRICE et al., 2011; NEUGEBAUER et al., 2012). MscL is made up of 136 residues and comprises two TM domains, interconnected by a periplasmic loop, and possesses a short α-helical domain inside the cytoplasm (CHANG et al., 1998). The identification of MscL protein to be a YidC substrate and the requirement of YidC for the assembly of the pentameric channel is discussed controversially (POP et al., 2009; PRICE et al., 2011; BERRIER et al., 2011; NEUGEBAUER et al., 2012). Recently, a YidC dependency on the membrane insertion of TssL, a tail-anchored membrane protein and component of the TypIV secretion system of enteroaggregative *E. coli* (EAEC), has been reported (ASCHTGEN et al., 2012; SOUSSOULA & KUHN, unpublished data).

A comparison of the so far identified YidC substrates reveals commonalities which seem to be general criteria for YidC dependency. All proteins are relatively small (approximately 10 kDa), not exceeding two TM domains and with short hydrophilic domains. A global approach to analyze the inner membrane proteome upon YidC depletion clearly has shown that only the biogenesis of membrane proteins with soluble domains smaller than 100 residues has been impaired (WICKSTRÖM et al., 2011b). A second specific characteristic of YidC substrates is most likely the distribution of negative charges in the periplasmic TM flanking regions or even in the TM domains itself (DALBEY et al., 2014). Pf3 coat protein carries two negative charged Asp residues at position 7 and 18 in the N-terminal region which is translocated into the periplasm during insertion. The same applies for F_oc; in this case the N-terminus destined to be located in the periplasm possesses single Glu and Asp residues. In addition, the periplasmic loops of M13 procoat and MscL are net negatively charged (-3 and -1, respectively). Regarding the insertion of an artificial model protein which does not require YidC and the Sec translocon it has been shown clearly that the addition of

negative charges to the translocated region results in YidC dependency (ZHU et al., 2013). Furthermore, the distribution of charged residues inside a TM domain might act as a determinant for the YidC pathway (PRICE & DRIESSEN, 2010; ZHU et al., 2013a). In a recent study, putative new endogenous *E. coli* YidC substrates have been identified. Interestingly, the above described criteria of negative charges were not found, but a rather unbalanced distribution of positive charges on both sides of the TM domains (GRAY et al., 2011). This was assigned to be a determinant for YidC dependency. Another criterion for YidC dependency might be a moderate hydrophobicity of the prospective substrate TM domain (ERNST et al., 2011; ZHU et al., 2013a).

With the extended use of global approaches to uncover cellular consequences upon YidC depletion a huge number of potential YidC substrates has been identified recently (PRICE et al., 2010; WANG et al., 2010; WICKSTRÖM et al., 2011b; GRAY et al., 2011). Interestingly, the putative substrates are not limited to small single or double-spanning proteins (GRAY et al., 2011). This is in accordance with results from *in vitro* studies suggesting that polytopic membrane proteins like MtlA can use solely either YidC or the Sec translocon for their membrane integration (WELTE et al., 2012).

1.4.4.1 Various pathways enable targeting to the YidC insertase

A general targeting pathway, which guides prospective substrate proteins to the YidC insertase, does not exist. Several observations suggest that there is an existing SRP/YidC pathway. It has been shown that the insertion of a constructed model membrane protein was inhibited upon SRP and YidC depletion (FRÖDERBERG et al., 2003). In addition, an *in vitro* study with inverted inner membrane vesicles (INVs) containing YidC with an incorporated cross-linker revealed contacts between the C-terminus of YidC, Ffh and FtsY (WELTE et al., 2012). The SRP-dependency of MscL targeting has been shown *in vivo* by abolishing the translocation of the periplasmic loop of MscL in the absence of Ffh and FtsY (FACEY et al., 2007; POP et al., 2009; NEUGEBAUER et al., 2012). Contradictory results have been obtained for F_oc. Here, it has been shown *in vitro* that the SRP system is not required for membrane insertion (VAN DER LAAN et al., 2004). This was confirmed in Ffh depleted cells expressing a F_oc variant with a His_{10} tag in the cytoplasmic loop and an additional tag fused to the very C-terminus (GVQDFTST) (YI et al., 2004). On the other hand the expression of a F_oc variant with a C-terminal HA tag (YPYDVPDYA) revealed a

SRP dependency of F_oc insertion (VAN BLOOIS et al., 2004). Furthermore, a contact of nascent TM1 to Ffh has been detected in this study.

It is generally accepted that targeting of the phage proteins Pf3 coat and M13 procoat occurs independently of the SRP system (CHEN et al., 2002; DE GIER et al., 1998), although Pf3Lep (a Pf3 coat variant with the P2 domain of leader peptidase fused to the C-terminus) and M13 procoat could be cross-linked to Ffh (CHEN et al., 2002; VALENT et al., 1995) (Figure 1.17). For both phage proteins targeting based on electrostatic interactions with the negatively charged phospholipid head groups is proposed. This has directly been shown for M13 procoat which exposes positively charged residues at the N- and C-Terminus (GALLUSER & KUHN, 1990). Pf3 coat protein contains two positive residues at its C-terminus (Figure 1.16). Mutation of these residues to negatively charged residues led to strongly reduced membrane association (KUHN, 1995). Thus, a similar targeting of Pf3 coat protein based on electrostatic interactions is assumed.

In addition, it is possible that cytosolic domains of YidC recognize and directly bind positively charged residues of substrate proteins (Figure 1.17). This has been shown for F_oc in co-sedimentation studies under YidC depletion conditions, suggesting only weak interactions of F_oc with negatively charged phospholipid head groups (KOL et al., 2008).

1.4.4.2 Pf3 coat protein is a model substrate for YidC mediated insertion

Pf3 coat protein is the major coat of filamentous bacteriophage Pf3 infecting Gram-negative *Pseudomonas aeruginosa* (STANISICH et al., 1974). Approximately 2500 copies of Pf3 coat together with minor coat proteins at the tips form a helical capsid of approximately 6 nm in diameter and up to 1 µm in length to cover the single-stranded DNA (ssDNA) of 5833 bases (WELSH et al., 1998). After insertion of Pf3 coat into the host membrane, the monomers oligomerize to encircle the ssDNA, which extrudes out of the cell as it is proposed for filamentous phage assembly (RUSSEL, 1991). Prior to the assembly, the secondary structure of Pf3 coat is approximately 40-75 % α-helical and folds almost completely to an α-helical structure onto phage progeny (AISENBREY et al., 2006; WELSH et al., 1998).

Pf3 coat protein is a type I membrane protein consisting of 44 AAs. The hydrophobic transmembrane region (18 AAs) is flanked by a negatively charged N-terminal region (18 AAs) and a short positively charged C-terminal tail of 8 AAs (Figure 1.16). As

mentioned above the insertion of Pf3 coat does not require SecA and SecYEG but strictly depends on the YidC insertase (ROHRER & KUHN, 1990; CHEN et al., 2002).

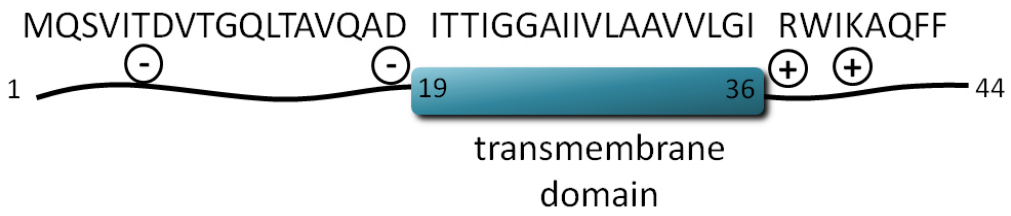

FIGURE 1.16 **Pf3 coat protein - a model substrate for YidC-mediated insertion.** Schematic representation, amino acid sequence and charge distribution of Pf3 coat.

1.4.4.3 YidC in action: Molecular mechanism of membrane insertion

The existence of both post-translational and co-translational YidC insertion pathways has been suggested (Figure 1.17). A post-translational insertion is proposed for M13 procoat and Pf3 coat due to their small size (DE GIER et al., 1998; CHEN et al., 2002). The cryo-EM structure of YidC bound to a translating ribosome (KOHLER et al., 2009) together with observed cross-links between ribosomes, Ffh and FtsY (WELTE et al., 2012) in an *in vitro* approach support a co-translational mechanism as it has been shown in yeast mitochondria by Oxa1 (JIA et al., 2003) and for a chimera YidC protein (SEITL et al., 2013; WICKLES et al., 2014).

The membrane biogenesis of Pf3 coat protein has been studied extensively to uncover the underlying molecular mechanism of YidC mediated insertion. It has been shown that during insertion YidC meets its substrates in the conserved core region (CHEN et al., 2002; YU et al., 2008; NEUGEBAUER et al., 2012). So far, direct substrate contacts to the non-conserved cytoplasmic regions of YidC have not been observed. Based on tryptophan fluorescence it has been shown that binding of Pf3 coat to YidC induces conformational changes within the periplasmic domains (WINTERFELD et al., 2009; IMHOF et al., 2011). Structural flexibility of the conserved periplasmic loops P2 and P3 is most likely a prerequisite for proper insertion of substrates. An exception is the P1 loop, which can be deleted almost completely without loss of function (JIANG et al., 2003).

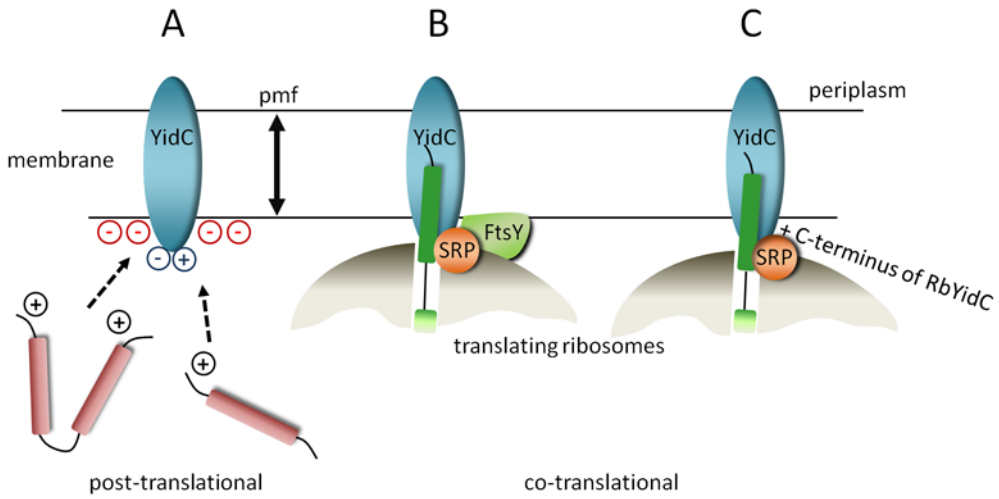

FIGURE 1.17 **Targeting of proteins to the YidC insertase and two modes of insertion. (A)** Post-translational insertion of substrate proteins - this has been shown for M13 procoat and Pf3 coat (DE GIER et al., 1998; CHEN et al., 2002). Most likely both proteins are targeted to YidC by electrostatic interactions between positively charged residues at the N- or/and the C-terminal end (black circles) and with negatively charged phospholipid head groups (red circles) (GALLUSER & KUHN, 1990; KUHN, 1995). In addition, a direct electrostatic attraction to the cytoplasmic regions of YidC is conceivable (blue circles). The insertion of both proteins requires the proton motive force (pmf) (KUHN et al., 1990; ROHRER & KUHN, 1990). **(B)** Co-translational insertion and targeting via SRP. RNCs of substrates are targeted to YidC by the SRP system, the insertion occurs in a co-translational way. One example is most likely MscL protein (FACEY et al., 2007). **(C)** The C-terminus of *Rhodopirellula baltica* YidC (RbYidC) fused to *E. coli* YidC can partially substitute for the SRP receptor FtsY and enables a direct contact to a translating ribosome (SEITL et al., 2013) as it was shown for the YidC homolog Oxa1 (JIA et al., 2003).

It was suggested that the translocation of the hydrophilic N-terminus of the Pf3 coat protein and the insertion of the transmembrane segment is driven by hydrophobic forces; based on the observation that altering the pH value during fluorescence measurements did not influence substrate binding (GERKEN et al., 2008). For translocation of highly negatively charged substrate domains the pmf is required to support YidC mediated insertion, as it has been found for M13 procoat and Pf3 coat (KUHN et al., 1990; ROHRER & KUHN, 1990; KIEFER et al., 1997). The insertion of MscL, F_oc and CyoA does not require the pmf (FACEY et al., 2007; VAN DER LAAN et al., 2004; CELEBI et al., 2006).

The conserved TM3 of YidC seems to play a predominant role in the insertion mechanism because various proteins, including Pf3 coat, M13 procoat, MscL and FtsQ, contact TM3 during their membrane insertion (CHEN et al., 2002; NEUGEBAUER et al.,

2012; WICKLES et al., 2014). In addition, the transmembrane segment of inserting FtsQ has been most likely found as an electron density in a cryo-EM structure of YidC next to TM3 (WICKLES et al., 2014).

A breakthrough in understanding the molecular mechanism of YidC mediated insertion was achieved recently by solving the x-ray structure of *Bacillus halodurans* YidC2 (KUMAZAKI et al., 2014a) and the creation of an accurate structural model of *E. coli* YidC (WICKLES et al., 2014). Based on the crystal structure and biochemical data it is suggested that a conserved positively charged arginine residue inside the discovered hydrophilic groove attracts negatively charged regions of substrate proteins. This allows an initial, partial insertion of the TM domain into the lipid bilayer. Indeed, the membrane protein insertion and folding monitor protein MifM (CHIBA et al., 2009), a single spanning substrate protein of YidC2, was successfully cross-linked into the hydrophilic groove (KUMAZAKI et al., 2014a). A step-wise neutralization of the acidic N-terminal MifM region (net charge -3 in the wild type) led to a decreased insertion efficiency until insertion was almost completely abolished (charge 0). Interestingly, when the acidic residues of a Pf3 coat/MifM fusion protein were mutated, the effect was less pronounced. The crucial role of the charged N-terminal domain for Pf3 coat insertion has already been shown (KIEFER et al., 1997). It is proposed that the pmf and/or hydrophobic interactions between the TM domain and the membrane core facilitate the translocation of the hydrophilic domain after substrate binding to the hydrophilic cleft. How this exactly happens is still not clear, because the hydrophilic groove penetrates deep into the YidC core domain and is closed towards the periplasm by the most rigid parts of the TM domains.

The mechanism described above fits well to single-spanning membrane proteins with a negatively charged N-terminus. However, the mechanism is based on a conserved arginine residue within the hydrophilic groove. In *E. coli* YidC, the corresponding residue R366 is not essential for function (JIANG et al., 2003) and only substitution to negatively charged residues inhibit the insertion of Pf3-Lep and to some extent of a M13 procoat variant (CHEN et al., 2014) . In addition, it remains unclear how double-spanning or even polytopic membrane proteins are inserted via YidC. Thus, it is conceivable that the insertion mechanism slightly differs among the YidC homologs and that some YidC substrates need other requirements for an efficient insertion.

1.4.4.4 YidC cooperates with the Sec translocon

At almost the same time when YidC has been uncovered as a membrane insertase, it was initially found that YidC is associated in a complex of SecYEG and SecDFYajC (SCOTTI et al., 2000). Subsequently, it was shown that YidC binds via SecD and SecF to the Sec translocon (NOUWEN & DRIESSEN et al., 2002; XIE et al., 2006). It is generally believed that YidC assists in the TM insertion and assembly of polytopic membrane proteins once they have exited the Sec complex through the lateral gate (SCOTTI et al., 2000; URBANUS et al., 2001; BECK et al., 2001; HOUBEN et al., 2004) (Figure 1.18). Indeed, YidC contacts TM helices in both halves of SecY which constitute the proposed lateral gate at the front side of the channel (SACHELARU et al., 2013). In addition, the dynamics of the SecY-YidC interaction have been shown, based on the finding that the binding sites vary in the presence or absence of active ribosomes displaying a nascent chain of FtsQ (SACHELARU et al., 2013).

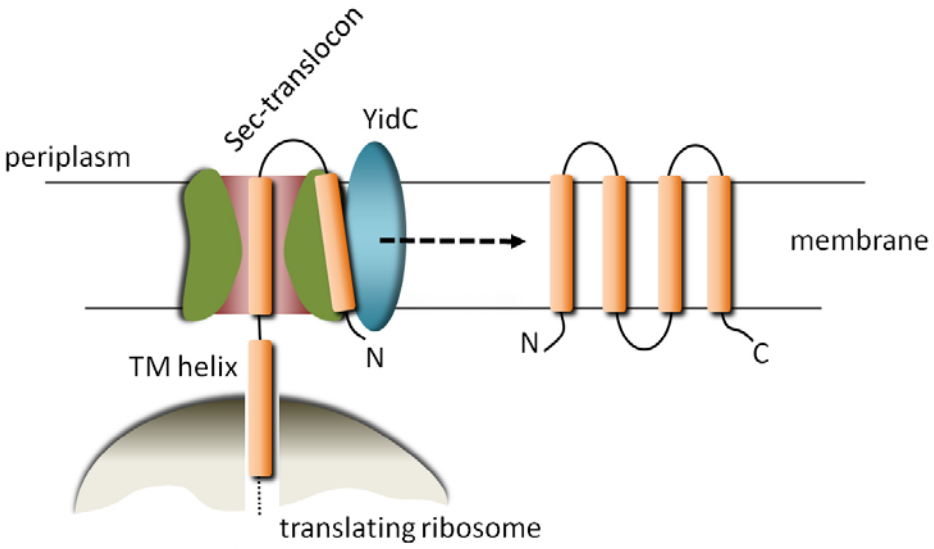

FIGURE 1.18 **YidC cooperates with the Sec translocon in membrane protein biogenesis.** Some but not all Sec-dependent membrane proteins require YidC for membrane insertion. Sec-dependent insertion of membrane proteins occurs mostly co-translationally and the TM helices of the nascent chain leave the channel sequentially through the proposed lateral gate. In the vicinity of the lateral gate YidC provides an assembly site for released TM domains and assists in folding and assembly prior to the final insertion.

Thus far, only a handful of proteins, including both polytopic and single-spanning proteins, have been shown to require strictly both YidC and the Sec translocon for insertion. Namely, these are the subunit a of the cytochrome bo_3 oxidase (preCyoA) (DU PLESSIS et al., 2006; CELEBI et al., 2006; VAN BLOOIS et al., 2006), subunit a and b (F_oa and

F_ob) of the F_1F_o-ATP synthase (KOL et al., 2009; YI et al., 2003; YI et al., 2004), and the subunit K of the NADH:ubiquione oxidoreductase (NuoK) (PRICE & DRIESSEN, 2010) (Figure 1.19). For the membrane biogenesis of preCyoA YidC acts upstream of the Sec translocon and facilitates insertion of the helical hairpin consisting of the signal sequence and the first TM domain. For the insertion of the second TM domain and the large periplasmic domain a coordinated action of SecYEG and SecA is required (CELEBI et al., 2006; VAN BLOOIS et al., 2006). A downstream action of YidC is conceivable in two different ways. Firstly, YidC is believed to provide an assembly platform prior to the sequentially release of TM segments into the bilayer, as it was shown for leader peptidase (HOUBEN et al., 2004). Secondly, YidC assists in the assembly of released TM domains to helical bundles prior to the insertion into the membrane (BECK et al., 2001; WAGNER et al., 2008).

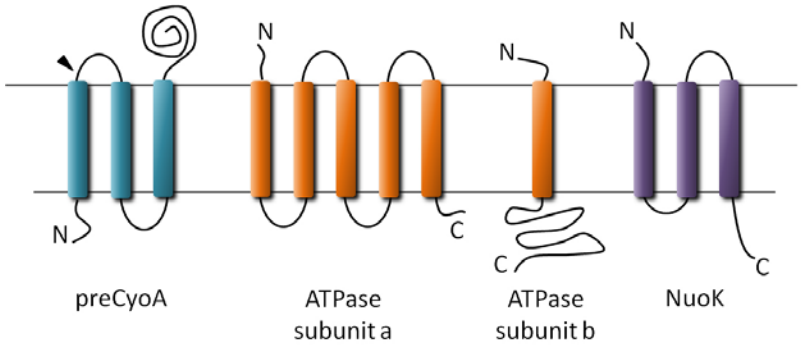

FIGURE 1.19 **Topology of membrane proteins that require both YidC and the Sec translocon.** YidC cooperates with the Sec translocon for the insertion of polytopic and single-spanning membrane proteins. PreCyoA (subunit a of the cytochrome bo_3 oxidase) is synthesized with an N-terminal signal sequence which is cleaved after insertion (the black arrowhead indicates the cleavage site).

For many Sec-dependent membrane proteins the orchestrated action of SecY and YidC seems not to be essentially required although a close proximity of YidC and the inserting polypeptides has been reported (WELTE et al., 2012; VAN DER LAAN et al., 2004). This suggests that YidC has an effect on the kinetics of the lateral release of polar domains out of the channel and might be required for efficient assembly and folding of the released substrates.

It is not clear how it is decided whether a Sec-dependent protein requires the function of YidC or not. However, it is suggested that the hydrophobicity of the TM domains and the distribution of charged residues within the hydrophobic domains or the periplasmic

regions determine the YidC dependency (PRICE & DRIESSEN, 2010; NEUGEBAUER et al., 2012; ZHU et al., 2013a; SOMAN et al., 2014).

1.4.4.5 YidC as a membrane embedded molecular chaperone

There are strong hints supporting the idea that YidC might function as a membrane embedded chaperone to assist folding of polytopic membrane proteins into their final tertiary conformation (DALBEY & KUHN, 2004). Lactose permease (LacY), consisting of 12 transmembrane helices in *E. coli*, recruites SRP/FtsY and the Sec translocon for membrane targeting and insertion (MACFARLANE & MÜLLER, 1995; SELUANOV & BIBI, 1991; ITO & AKIYAMA, 1991). Although YidC is not required for membrane insertion per se, a direct role of YidC in folding of LacY has been shown (NAGAMORI et al., 2004). Folding of LacY in YidC depleted membrane vesicles leads to structural alterations which were indirectly detected with conformational monoclonal antibodies, binding native folded epitopes on both sides of the membrane (NAGAMORI et al., 2004). In addition, *in vivo* studies suggest, that periplasmic loops and helix packing of LacY both are disordered in the absence of YidC (ZHU et al., 2013b). Similar results have been found for the protein MalF, a subunit of the MalFGK maltose transporter. Here, the stability of MalF and even the assembly of the entire MalFGK complex are significantly reduced in cells depleted of YidC (WAGNER et al., 2008). The upregulation of chaperones as a cellular response to overcome YidC depletion further support the role of YidC as a chaperone involved in the folding and assembly of TM domains and folding of hydrophilic loops. YidC might also be involved in the degradation machinery to remove misfolded proteins from the membrane, as YidC was successful cross-linked to a large complex of membrane bound chaperons and proteases FtsH, HflC and HflK (VAN BLOOIS et al., 2008).

Interestingly, YidC appeared to be important for the biogenesis of Sec-dependent secretory proteins and lipoproteins as well. For the autotransporter hemoglobin protease (Hbp), a virulence factor secreted by Gram-negative bacteria, it was shown that the Hbp signal peptide contacts YidC during early biogenesis. It is suggested that YidC chaperones Hbp into a secretion competent state in the periplasm after its translocation (JONG et al., 2010). The specific role of YidC in the biogenesis of the lipoproteins Lpp (murein lipoprotein) and BRP (bacteriocin release protein) remains unclear but it is assumed that YidC is required for the insertion of the signal peptides and/or fot chaperoning Lpp and BRP to the Lol-system (FRÖDERBERG et al., 2004).

1.4.4.6 YidC assists in the assembly of highly ordered membrane protein complexes

The function of YidC to work as an insertase and molecular chaperon is related to its involvement in the assembly of membrane protein complexes. In comparative analyses of oligomeric membrane protein complex levels in *E. coli* cells containing YidC or not, it was clearly shown that YidC depletion leads to reduced levels of these complexes (WICKSTRÖM et al., 2011). Specifically, the assembly of the F_1F_o-ATP synthase, the cytochrome bo_3 oxidase and the NADH dehydrogenase was reduced by approximately 60 %, approximately 50 % and approximately 30 %, respectively (WICKSTRÖM et al., 2011). As mentioned above, YidC is crucial for the insertion of subunits of all these membrane protein complexes. This implies a related function of YidC for the assembly of respiratory chain and energy-transducing complexes as it was found in yeast mitochondria for the F_1F_o-ATP synthase (JIA et al., 2007). The co-purification of YidC and subunits of the NADH dehydrogenase, the cytochrome bo3 oxidase and F_oc from the F_1F_o-ATP synthase (PRICE et al., 2010; KOL et al., 2008) and the fact that SpoIIIJ and YqjG, YidC homologs from *Bacillus subtilis*, copurify with the F_1F_o ATP synthase complex, further support this idea (SALLER et al., 2009).

Objectives of this thesis

The membrane insertase YidC inserts newly synthesized proteins into the plasma membrane. YidC protein of *Escherichia coli* is a six-spanning protein of 548 amino acid residues with a large periplasmic domain between the first two TM regions. YidC is required for the biogenesis of respiratory complexes, ATP synthase and for example the mechanosensitive channel protein MscL. Also, the coat proteins of filamentous phages Pf3 and M13 strictly require YidC for membrane insertion.

The best studied YidC substrate is the 44 amino acid residues long Pf3 coat protein of the phage Pf3, infecting *Pseudomonas aeruginosa*. During the insertion process, the Pf3 coat protein binds reversibly to YidC and adopts finally a transmembrane conformation.

The aims of this study were the identification of YidC-substrate contacts during membrane biogenesis of the inserting Pf3 coat protein and the investigation of the molecular mechanism and the dynamics underlying YidC-mediated insertion. In order for this to be achieved, a set of single cysteine mutants of YidC and the inserting Pf3 coat protein were prepared. Next, an *in vivo* cross-linking assay was established to monitor YidC-substrate complex formation during the insertion process. Attempts were made to detect substrate binding in distinct regions of the YidC TM domains.

In addition, this study aims to characterize the insertion process of translocation deficient Pf3 coat mutants. The translocation of these Pf3 coat variants was blocked due to charge changes in the N-terminal segment flanking the transmembrane domain. Dynamic insertion studies allow determining whether these mutants still contact YidC in an initial state of the partial membrane insertion.

Chapter 2

The Pf3 coat protein contacts TM1 and TM3 of YidC during membrane biogenesis

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ABSTRACT

The coat protein of bacteriophage Pf3 is inserted into the plasma membrane of Escherichia coli by the insertase YidC. To identify which of the six transmembrane regions of YidC bind the single-spanning Pf3 coat protein during membrane protein biogenesis, we used the disulfide cross-linking approach. We generated single cysteines in each of the transmembrane regions of YidC and in the center of the hydrophobic region of Pf3 coat protein. We found that the substrate Pf3 coat contacts the first and third transmembrane segment (TM) of YidC as cross-links between these two proteins can be formed in vivo during membrane biogenesis. A detailed disulfide-mapping study revealed that one face of TM3 of YidC makes contact with the Pf3 protein.

INTRODUCTION

In bacteria, membrane proteins are inserted into the membrane by two unrelated evolutionarily conserved pathways. The main pathway requires the protein-conducting SecYEG channel and accessory components SecDFYajC, YidC, and SecA for membrane protein insertion (for review see [1,2]). SecY and SecE are related to Sec61a and Sec61c in the ER system. The other pathway requires the membrane protein insertase YidC for

membrane insertion of Sec-independent substrates (for review, see [3–5]. YidC is homologous to Oxa1 and Alb3, respectively, used to insert proteins into the mitochondrial inner membrane and the plant thylakoid membrane [5].

In both the SecYEG and the YidC only pathways, YidC comes into contact with the transmembrane segments of membrane protein substrate during the insertion process [6–8]. YidC is critical for membrane protein insertion of Sec-independent substrates that use the ''YidC only pathway'' [9–11]. YidC only substrates include the M13 procoat, Pf3 coat, and F_o subunit c of the F_1F_o ATPase and the MscL protein [11–15]. YidC substrates that go by the Sec pathway include subunit a of the F_1F_o ATPase, subunit II of the cytochrome bo oxidase, and Lac permease [16–20]. For Lac permease, YidC is believed to function as a chaperone for the folding of the protein.

YidC family members all possess significant sequence homology in the C-terminal region that contains five transmembrane segments [21]. The C-terminal five transmembrane segments, in particular transmembrane segment 3, are important for function [22,23]. The C-terminal transmembrane regions of the YidC protein may comprise a platform that binds the substrate and then promotes the insertion of the hydrophobic region perpendicular to the membrane bilayer [24].

In this paper, we examine which regions of YidC contact the substrate during membrane biogenesis. The Pf3 coat protein was used as the substrate because it is known to cross-link to YidC in vitro [13]. Using disulfide-mapping, we found that we could cross-link the Pf3 coat to YidC via single cysteines located in the transmembrane region of Pf3 coat and in the transmembrane region of YidC in TM1 and TM3.

MATERIALS AND METHODS

E. coli JS7131 and MK6 were transformed with pMS-Pf3 coat and pGZ-YidC and then grown in M9 minimal media in the presence of ampicillin (final concentration, 100μg/ml) and chloramphenicol (final concentration, 25μg/ml). Depletion of the chromosomally-encoded YidC was carried out by growth in the presence of 0.2% glucose for 3 h at 37°C. Unless indicated otherwise, the cells were treated with 1 mM isopropyl-thiogalactoside (IPTG) at OD_{600} nm 0.2 for 10 min to induce expression of Pf3 coat and YidC, and pulse-labelled for the indicated time by the addition of [^{35}S]-

methionine. The cells were chilled on ice and if indicated, treated with 1 mM freshly prepared copper 1,10-phenanthroline for 10 min [25]. The samples were precipitated with 20% trichloroacetic acid (TCA), washed with acetone, and resuspended in 2% SDS in Tris-buffer, pH 8. Immunoprecipitation was done as described [26] using antiserum to Pf3 virus, his-tag, OmpA, and leader peptidase. For complementation assays, the JS7131 strain was transferred with pACYC184 vector with the YidC gene under the control of its endogenous promoter [27]. For protease protection analysis the cells were converted to spheroplasts as described [25] and treated with 0.5 mg/ml proteinase K for 1 h on ice. The site-directed mutations in Pf3 coat and YidC were generated by the Quik-Change method with minor modifications [28]. All mutations were verified by DNA sequencing of the entire gene.

RESULTS

Single cysteine mutants in Pf3 coat and YidC

To investigate which of the regions of YidC contact the Pf3 coat substrate during membrane biogenesis, we used an in vivo cysteine cross-linking approach. In this approach, a unique cysteine is placed in both the substrate and the membrane insertase YidC. We created two single cysteine mutants of Pf3 coat in the transmembrane region of the protein substrate and 16 single cysteine mutants of YidC by site-directed mutagenesis (Figure 1).

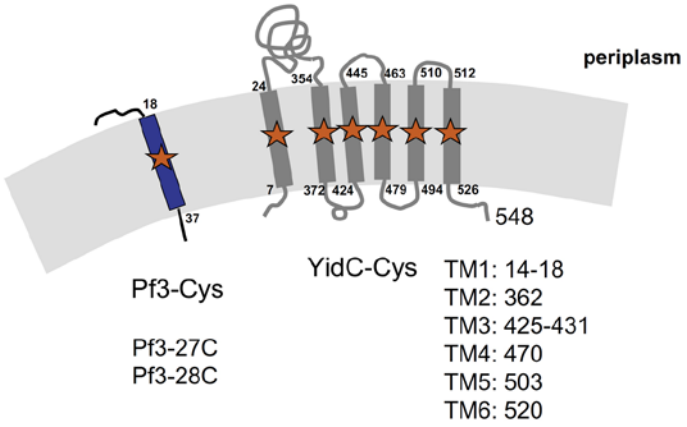


FIGURE 1 **Schematic representation of YidC and Pf3 coat protein and their topology in the inner membrane of *E. coli*.** The predicted membrane spanning segments (TM) are indicated by a rectangle and the amino acid residue numbers flanking the TM region indicated. The position of the single cysteine mutations are highlighted with a star.

The Pf3 mutations replaced the isoleucine and valine at positions 27 and 28, respectively. Expression of these Pf3 coat mutants was inducible by adding IPTG to the growth medium, since the Pf3 genes were cloned into the plasmid pMS119HE [29]. In YidC, a cysteine residue was placed into the center of each of the six transmembrane regions at positions 16, 362, 430, 470, 503 and 520 starting with a protein that lacks the endogenous cysteine at position 423 (cysteine-less background). The YidC mutant genes were cloned either into pACYC184 (for growth complementation) or the pGZ119EH plasmid (for membrane protein insertion assay). Both of these plasmids are compatible with the pMS plasmids [29].

Functionality of the YidC mutants with single cysteines

The functionality of the YidC mutants was determined by testing whether the single cysteine YidC mutants could complement the YidC depletion strain JS7131. JS7131 has the YidC gene under the control of the araBAD promoter cloned into the lambda attachment site and the endogenous YidC gene was inactivated by a deletion [9]. This strain is arabinose dependent for growth. JS7131 was transformed with the various YidC mutants in pACYC184 and the strain streaked onto a LB agar plate containing either 0.2% arabinose (Figure 2A, Ara) or 0.2% glucose (Figure 2A, Glc). Figure 2A shows that all the YidC cysteine mutants could complement the growth defect of the JS7131 strain under glucose conditions where the chromosomal YidC expression is repressed. Only the negative control where the JS7131 strain was transformed with the pMS119 plasmid could not grow on LB agar plates with 0.2% glucose.

Protease mapping studies also verified that the single cysteine YidC mutants were fully functional as a membrane protein insertase. For these studies, we transformed the YidC mutants into the arabinose-dependent MK6 strain where, like the JS7131 strain, the expression of the chromosomal *yidC* is under the control of the araBAD promoter but the gene is left at its original position in the chromosome. The YidC cysteine mutants were coexpressed with the Pf3-P2 coat protein that encodes an extended version of the Pf3 coat protein at the C-terminus to allow immunoprecipitation of the proteinase K protected fragment (Figure 2B). MK6 cells with both plasmids were grown for 3 h in medium containing 0.2% glucose to deplete the chromosomal encoded YidC protein. Radiolabelled cells expressing the YidC mutants and Pf3 coat were subjected to proteinase K mapping (see Figure 2 legend for details). Significantly, when the single

cysteine mutants were expressed, efficient insertion of Pf3-P2 does occur as Pf3-P2 is converted to the shifted form by protease cleavage (see lanes 5–16), but not when cells without plasmid were depleted of YidC (lane 4; non-digested form of Pf3-P2 accumulated). These studies verified that the YidC mutant proteins are functional.

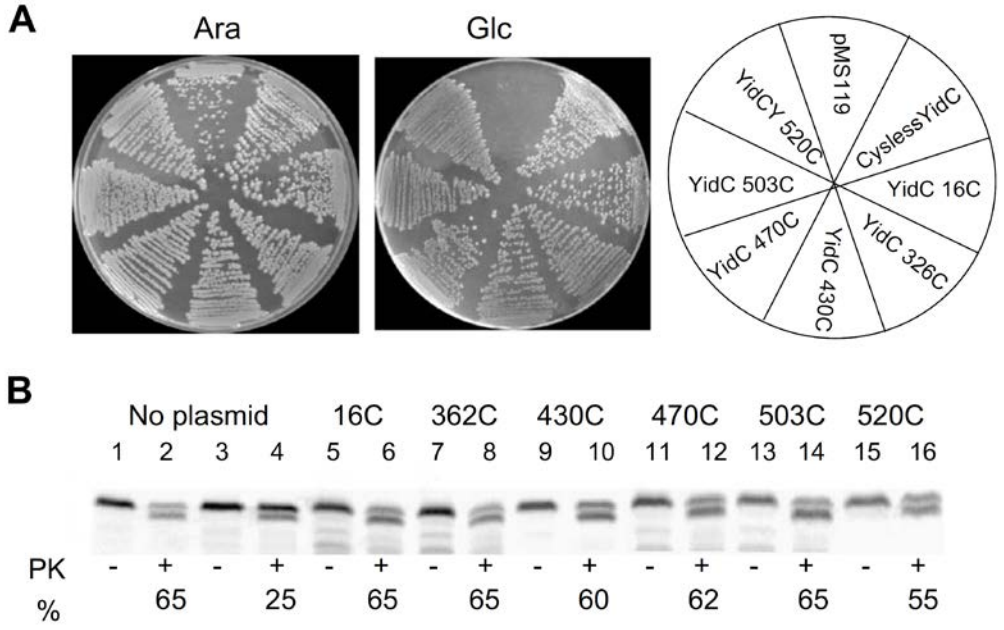


FIGURE 2 **Functionality of the YidC cysteine mutants. (A)** Complementation of growth of the YidC depletion strain MK6 transformed with plasmids pACYC184 (vector without insert), or with plasmid expressing the YidC423S (cysteine-less YidC), YidC16C, YidC326C, YidC430C, YidC470C, YidC503C, YidC520C mutants, where the number indicates the position of the single cysteine. **(B)** Membrane insertion of Pf3-P2 in MK6 cells bearing the IPTG inducible pMS119 vector containing Pf3-P2 and pGZ119 vector containing the single cysteine YidC mutant. The cells were induced for 10 min with 1 mM IPTG to express Pf3-P2 and the YidC cysteine mutants followed by pulse-labelling with [^{35}S]-methionine for 1 min, then chased with non-labelled methionine for 1 min and converted to spheroplasts. One aliquot was treated with proteinase K for 60 min on ice (+PK, even lanes), whereas another aliquot was untreated (-PK, odd lanes). The cells were TCA-precipitated and analyzed by SDS–PAGE and phosphorimaging. The percentage of digested Pf3-P2 coat protein was determined. The cells without a YidC encoding plasmid were grown in 0.2% arabinose (lanes 1 and 2) or 0.2% glucose (lanes 3 and 4). Cells bearing the plasmids encoding for the respective YidC mutant were grown in 0.2% glucose (5–16).

Interaction between the Pf3 coat protein and YidC

To identify the transmembrane domain of YidC that contacts the Pf3 coat substrate during membrane biogenesis, we used disulfide-mapping. In this approach, a stable disulfide-linked YidC-substrate complex will form if the cysteines are in close proximity under oxidizing conditions. Coexpression of the Pf3 coat and YidC containing single

cysteines were analyzed under oxidizing conditions to test whether transmembrane segments of the substrate and YidC contact each other at the cysteine positions under oxidizing conditions (Figure 3). MK6 cells harbouring a plasmid coding for Pf3-27C and a plasmid coding for a single cysteine YidC mutant were grown at 37 °C for 3 h in the presence of glucose. The proteins were expressed by the addition of 1 mM IPTG for 10 min and then labeled by the addition of [^{35}S]-methionine for 1 min. Then, one aliquot was incubated with 1 mM copper phenanthroline (CuP) for 10 min at 0 °C (+CuP lanes), and another aliquot was placed on ice (-CuP). The samples were TCA-precipitated, resuspended in SDS, and immunoprecipitated with antiserum to Pf3 phage. The samples were then treated with DTT (-CuP) or not (+CuP) and analyzed on a non-reducing SDS–PAGE gel. As expected, the addition of the oxidizing agent CuP increases the amount of Pf3 coat dimer with a concomitant decrease in the amount of the Pf3 coat monomer (Figure 3; compare even and odd lanes). In addition, in the cells that express Pf3-27C coat and YidC with one cysteine in the trans- membrane region TM1 or TM3 we observed an additional band at about 65 kDa which was more intense under oxidizing conditions (lanes 4 and 8) compared to the reduced conditions (lanes 3 and 7). Under the reduced condition (lane 3) we observed a slightly lower band at about 60 kDa that probably corresponds to YidC that dissociated from Pf3 coat protein on the SDS–PAGE gel after remaining non-covalently bound during the immunoprecipitation. YidC-Pf3 cross-linking at 16 C and 430 C was also observed when Pf3-28C coat was coexpressed (lanes 15 and 16).

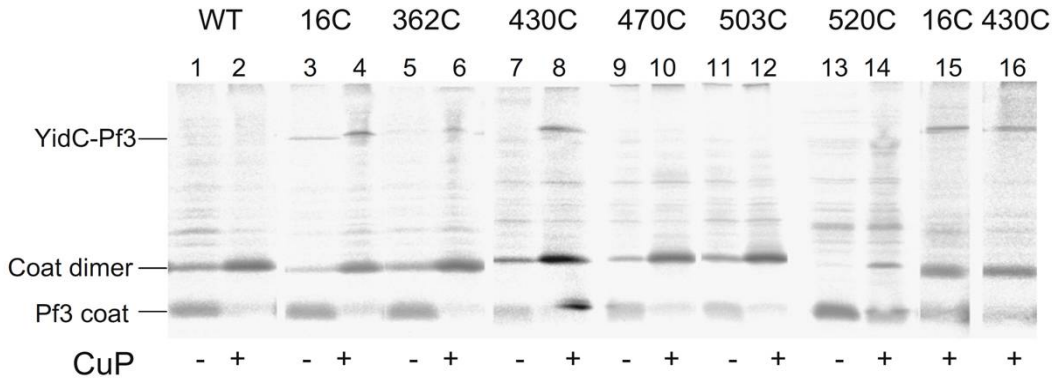


FIGURE 3 **The TM1 and TM3 of YidC contact the Pf3 coat protein.** MK6 cells bearing the plasmid pMS-Pf3-27C (lanes 1–14) or pMS-Pf3-28C (lanes 15 and 16) and a second plasmid, pGZ119 encoding the respective single cysteine YidC mutant were grown in the presence of 0.2% glucose to deplete the cells from the chromosomally-encoded YidC. The cells were induced with 1 mM IPTG for 10 min and pulse-labelled with [^{35}S]-methionine for 1 min. One aliquot was put on ice (-CuP, odd lanes), whereas the other aliquot was treated with 1 mM copper phenanthroline (+CuP, even lanes) for 10 min. The cells were TCA-precipitated, immunoprecipitated with antibody to the Pf3 virus, and analyzed on a non-reducing SDS–PAGE and phosphorimaging.

To verify that cross-linking occurs between Pf3 coat and certain YidC cysteine mutants, we tested whether the 65 kDa band is immunoprecipitated with an antibody directed to the 10his-tag located at the C-terminus of the YidC-Cys mutant proteins (Figure 4A, lanes 2 and 4). Figure 4A shows with the Pf3 coat 27C and the YidC430C mutant that a 65 kDa band is detected with Pf3 coat antiserum (lane 3) and with the his antiserum (lane 4). The 65 kDa band that is recognized by the Pf3 and YidC antibodies has a slightly higher molecular weight than the 10hisYidC alone (lane 2). As expected, no Pf3 coat monomers or dimers are observed with the his antiserum (lane 4) while they are detected with Pf3 antiserum (lanes 1 and 3).

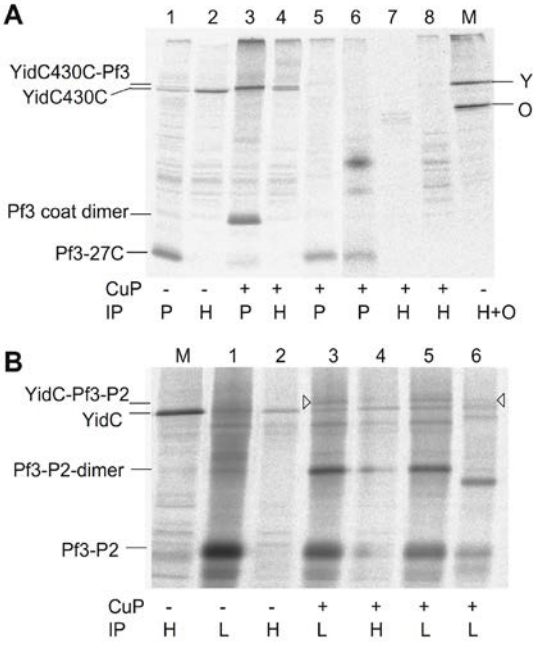


FIGURE 4 **Analysis of the Pf3-YidC cross-linked products. (A)** The cross-linked Pf3-27C – YidC430C is recognized by both antibodies and can be reduced. MK6 cells with the plasmids encoding Pf3-27C and YidC430C were pulse-labelled with [^{35}S]-methionine for 1 min, treated with copper phenanthroline for 10 min (+CuP, lanes 3–5) or were left untreated (-CuP, lanes 1 and 2). One oxidized aliquot was reduced by 1 mM DTT and 1 mM EDTA (lane 5). The samples were immunoprecipitated with antibodies to the Pf3 virus (P, lanes 1, 3, 5, and 6), and to the his10-tag (H, lanes 2, 4, 7, 8, and M). For a control, cells with only the plasmid encoding Pf3-27C (lanes 6 and 7) and cells with only the plasmid encoding YidC430C (lanes 8 and M) are shown. They were grown either in the presence of IPTG or without induction (lane 8). The sample in lane M was precipitated with antibodies to the histag (H) and to OmpA (O), showing YidC430C (Y) and OmpA (O) as size markers. **(B)** Cross-linking of YidC to Pf3-P2 that has an extended carboxyl-terminal domain. MK6 cells expressing YidC-430C was coexpressed with Pf3-P2 that had a cysteine at position 28. After pulselabelling for 1 min, the cells were chilled on ice and an aliquot was oxidized with CuP for 10 min (+CuP, lanes 3–6). Lanes 1 and 2 show the samples under non-oxidized conditions (-CuP). For the samples in lanes 5 and 6 the cells were converted to spheroplasts and 0.5 mg/ml proteinase K was added for 1 h (lane 6). The samples were TCA-precipitated and immunoprecipitated with antiserum to leader peptidase (L, lanes 1, 3, 5, and 6) and to his-tag (H, lanes M, 2 and 4). YidC has a his10-tag at the C-terminus. As a size marker, YidC430C is shown in lane M.

The oxidized samples were reduced by the addition of 1 mM DTT and 1 mM EDTA resulting in the dissociation of the YidC–Pf3 complex (lane 5). No YidC is detected with the Pf3 coat antiserum in this sample. As a control, cells expressing the Pf3-27C without a YidC-Cys mutant were analyzed (lanes 6 and 7). No protein was detectable at the position of 65 kDa. As a control, we show cells with only the YidC430C plasmid after induction with IPTG (lane M) and the non-induced cells (lane 8). For a size reference, the sample shown in lane M was precipitated with OmpA antiserum together with the antiserum to YidC-his.

Importantly, the substrate that is cross-linked to YidC during a 1 min pulse is not accessible to protease. We showed this using a Pf3 coat that was extended at the C-terminus by the P2 domain of leader peptidase. Figure 4B indicates that due to this extension the Pf3-YidC cross-linked product is now ~ 75 kDa in size (Figure 4B, lane 3, arrowhead). After oxidation, the cells were converted to spheroplasts and treated with proteinase K. The Pf3-P2 dimers were clipped by the proteinase K and converted to a protease-protected fragment that was recognized by the antibody to leader peptidase (Figure 4B, lane 6), demonstrating that the dimers were inserted across the membrane. The cross-linked Pf3-P2-YidC was largely resistant to the protease (Figure 4B, lane 6) suggesting that the N-terminal tail of Pf3-P2 was not fully accessible from the periplasmic side of the membrane.

To examine how the YidC TM1 and TM3 regions contact the Pf3 coat substrate, additional residues within TM1 were changed to a cysteine at positions 14–18 and in TM3 from residues 425 to 431. Coexpression with Pf3-27C coat protein showed that the interaction with YidC17C (Figure 5A) and with YidC430C (Figure 5B) is very specific. As a control, Pf3-16C, which has a single cysteine in the amino-terminal hydrophilic region, did not cross-link to YidC16C (Figure 5A; lane 6). Also, no cross-link was observed with Pf3-27C when YidC without a cysteine was coexpressed (lane 7). We verified that Pf3 coat and YidC-Cys mutants were expressed at a comparable level (data not shown).

DISKUSSION

In this paper, we show that TM1 and TM3 of YidC make contact with the Pf3 coat protein during membrane biogenesis. These contacts presumably occur in the substrate

binding pocket of YidC that is important for the membrane insertion reaction of the Pf3 coat protein. The failure of TM2, TM4, TM5 and TM6 to efficiently cross-link the Pf3 coat protein does not prove that these regions are not part of the binding pocket. It is possible that an unproductive positioning of the cysteines in these regions is the reason that no cross-linking had occurred. Further experiments are necessary to show whether they are part of the substrate binding pocket. Cross-links were observed between the first and third transmembrane segments of YidC and the transmembrane region of Pf3 coat upon adding the oxidant copper phenanthroline. These cross-links are specific since they did not occur under reducing conditions. The YidC/ Pf3 coat cross-link was immunoprecipitable with antiserum against Pf3 coat and against a his-tag that recognized the mutant YidC.

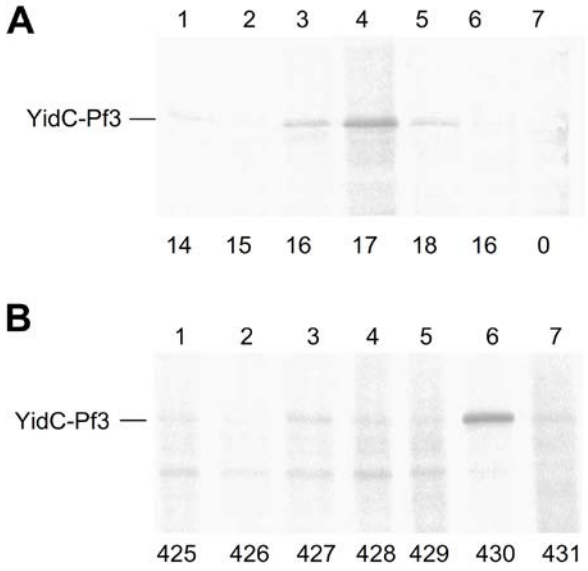


FIGURE 5 **The Pf3 substrate interacts with one helical face of YidC TM1 and TM3.** Single cysteine mutants of YidC were investigated for crosslinking with Pf3-27C **(A)** in TM1 of YidC at positions 14 (lane 1), 15 (lane 2), 16 (lane 3), 17 (lane 4), and 18 (lane 5). For a control, Pf3-16C was coexpressed with YidC16C (lane 6) and Pf3-27C was coexpressed with the cysteine-less YidC (lane 7). In TM3 **(B)**, residues 425 (lane 1), 426 (lane 2), 427 (lane 3), 428 (lane 4), 429 (lane 5), 430 (lane 6), and 431 (lane 7), were analyzed for crosslinking with Pf3-27C. The cells were induced with 1 mM IPTG for 10 min and pulse-labelled with [^{35}S]-methionine for 30 s and treated with 1 mM copper phenanthroline for 10 min. The cells were TCA-precipitated, immunoprecipitated with antibody to the his-tag recognizing the YidC mutants and analyzed on a non-reducing SDS–PAGE and by phosphorimaging. The intensity of the bands were quantified with the background subtracted and plotted (Supplementary Figure S1).

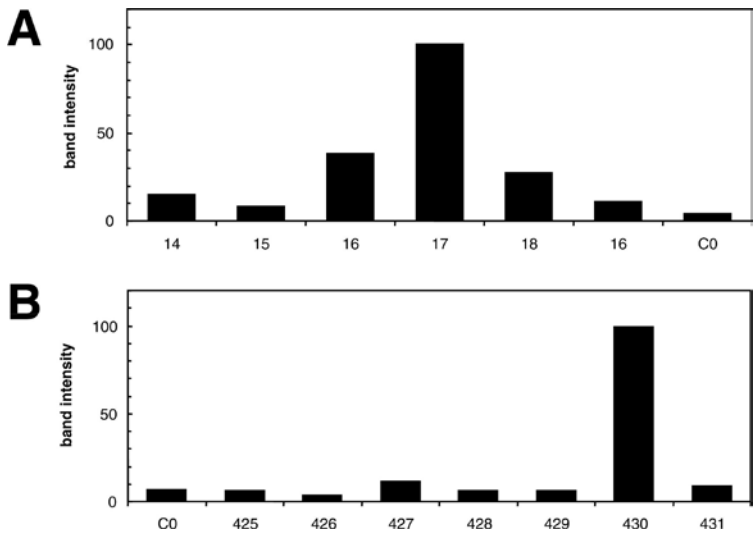
While TM1 is not of general importance to the YidC/Oxa1/Alb3 family of proteins since many members lack this transmembrane segment [21,30], TM3 is of functional importance. First, deletion of TM3 and TM4 and some single serine mutations in TM3

impairs YidC function [22]. In addition, TM3 (419PLGGCFPLLIQMPIFLALYYMLMG-SV444) contains part of the consensus sequence (see bold residues) within the YidC family of proteins. Finally, mutations (C423R and P431L) that confer cold-sensitive growth also are localized in the YidC TM3 [23].

Exactly what features of the substrate/membrane insertase are important for binding is not known. However, it seems probable that hydrophobic forces will be important for this process since the regions that make contact are hydrophobic in nature. In addition, it was found that the binding of YidC to Pf3 coat in detergent was only slightly affected by pH changes, suggesting that hydrophobicity might be a major force for binding to YidC [31]. Future studies using mutagenesis and cross-linking approaches will address this issue.

The interacting face of the transmembrane helices TM1 and TM3 were studied showing that the residues 16, 17 and 430 were cross-linked to the Pf3 substrate, residues 427 and 18 showed weak interactions, whereas residues 14, 15, 425, 426, 428, 429 and 431 were not cross-linked. Taken together, the data clearly show that only one face of TM3 of YidC is interacting with the Pf3 substrate protein.

SUPPLEMENTARY DATA



SUPPLEMENT FIGURE S1 **The intensity of the bands from Figure 5 A and B were quantified with the background subtracted and plotted.**

ACKNOWLEDGEMENTS

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CONTRUBTION TO THIS MANUSCRIPT

C.K. performed experiments uncovering YidC-Pf3 contacts. J.Y. performed experiments to verify the functionality of the YidC cysteine mutants. C.K., J.Y. and A.K prepared the figures. C.K., R.E.D. and A.K. wrote the manuscript. R.E.D. and A.K. supervised and directed the research.

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Chapter 3

Dynamic disulfide scanning of the membrane-inserting Pf3 coat protein reveals multiple YidC substrate contacts

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ABSTRACT

The membrane insertase YidC inserts newly synthesized proteins into the plasma membrane. While defects in YidC homologs in animals and plants cause diseases, YidC in bacteria is essential for life. Membrane insertion and assembly of ATP synthase and respiratory complexes is catalyzed by YidC. To investigate how YidC interacts with membrane-inserting proteins, we generated single cysteine mutants in YidC and in the model substrate Pf3 coat protein. The single cysteine mutants were expressed and analyzed for disulfide formation during 30 s of synthesis. The results show that the substrate contacts different YidC residues in four of the six transmembrane regions. The residues are located either in the region of the inner leaflet, in the center, as well as in the periplasmic leaflet, consistent with the hypothesis that YidC presents a hydrophobic platform for inserting membrane proteins. In a YidC mutant where most of the contacting residues were mutated to serines, YidC function was severely disturbed and no longer active in a complementation test, suggesting that the residues are important for function. In addition, a Pf3 mutant with a defect in membrane insertion was deficient to contact the periplasmic residues of YidC.

INTRODUCTION

The membrane insertase YidC of Escherichia coli is required for the biogenesis of respiratory complexes, ATP synthase, and other membrane proteins (1, 2). When YidC is depleted from bacterial cells, growth ceases, and the cells die, revealing that it is an

essential protein (3, 4). In the absence of YidC, the subunit c protein of the F_o part of the ATP synthase has been shown to accumulate in the cytoplasm because its membrane insertion is blocked (5, 6). Also a number of other proteins, such as the CyoA protein of the terminal oxidase complex (7), the mechanosensitive channel protein MscL (8), and the coat proteins of filamentous phage Pf3 and M13 require YidC for their membrane insertion. All these substrate proteins comprise less than 200 amino acids and contain periplasmic regions of less than 30 residues. The best studied substrate is the 44 amino acid-long Pf3 coat protein. After its synthesis, it binds reversibly to YidC and is membrane-inserted within a few minutes. The binding of purified Pf3 coat protein has been investigated by its ability to quench the 1-anilinonaphthalene-8-sulfonate (ANS)-labeled YidC protein in detergent (9) and also to quench the membrane-integrated insertase (10). The insertion of the Pf3 coat protein had been studied in a reconstituted system with proteoliposomes that only contained YidC (11). When limiting amounts of YidC were present in the proteoliposomes, up to 150 Pf3 coat proteins were inserted per YidC molecule, suggesting that YidC is catalytically driving the membrane insertion of proteins.

YidC is a six-spanning membrane protein of 548 amino acids (12). The structure of the large periplasmic domain between the first two transmembrane (TM) regions has recently been solved to high resolution (Figure 1) (13, 14). The homologues found in mitochondria and chloroplasts lack the first transmembrane region and most of the first periplasmic domain of 330 amino acid residues. Accordingly, the deletion of 300 residues from the periplasmic domain of the YidC from *E. coli* retained a still functional YidC mutant deficient for the interaction with SecF (15). When the first transmembrane region of YidC was replaced by a cleavable signal peptide, it maintained activity (16). However, the transmembrane regions were shown to be essential for the function of YidC. Deletions of TM3/4 and TM5/6 resulted in inactive YidC (17). Also, the replacement of residues 418 to 425 by serines did not retain activity. Surprisingly, a number of substitutions of residues in the transmembrane regions with serines or alanines had no noticeable effect (17). Presumably, a number of residues in the hydrophobic transmembrane regions of YidC are involved in substrate binding and are required during the insertion process.

The transmembrane regions are the most conserved parts of the YidC protein. Particularly, this was observed for the sequence ofTM2 andTM3 throughout and for the periplasmic portions of TM5 and TM6 (12). Single cysteines in TM3 were shown to be involved in substrate contacts in vivo (18) and in vitro (19). Here, we have extended this study and systematically scanned all six transmembrane regions of YidC for contacts with a substrate by single cysteine mutants. The results show that TM1, TM3, and TM4 provide nine major contacting residues. Three additional contacts are located in the periplasmic half of TM5. A substrate mutant that is deficient for membrane insertion was able to contact the YidC residues close to the cytoplasm but failed to contact the residues in the periplasmic half of the transmembrane region.

EXPERIMENTAL PROCEDURES

Bacterial Growth, Pulse Labeling, and Disulfide Formation

E. coli MK6 (18) was transformed with pMS-Pf3 coat and pACYC-YidC and then grown in M9 minimal media in the presence of ampicillin (final concentration, 100 μg/ml) and chloramphenicol (final concentration, 25 μg/ml). In the MK6 strain, the promoter of the yidC gene had been exchanged with the araC-araBAD promoter cassette. Depletion of the chromosomally encoded YidC was achieved by growth in the presence of 0.2% glucose for 3 h at 37 °C. Unless indicated otherwise, the cells were treated with 1mM isopropyl 1-thio-D-galactopyranoside (IPTG) at $A_{600\ nm}$ 0.2 for 10 min to induce expression of the Pf3 coat and pulse-labeled for the indicated time by the addition of [^{35}S]methionine. The cells were chilled on ice and, if indicated, treated with 1mM freshly prepared copper 1,10-phenanthroline for 3 min (20). The samples were precipitated with 20% TCA, washed with acetone, and resuspended in 2% SDS in Tris buffer (pH 8). Immunoprecipitation was done as described (21) using antiserum to Pf3 phage or to YidC. The samples were analyzed by SDS-PAGE and phosphor imaging.

Generation of Single Cysteine Mutants

The site-directed mutations in the Pf3 coat and YidC were generated by the QuikChange method with minor modifications (22). All mutations were verified by DNA sequencing of the entire gene.

Protease Mapping with Proteinase K

E. coli BL-21 (23) cells bearing the respective plasmids were grown overnight with 0.2% arabinose and diluted 1:100 into fresh minimal medium with 0.2% glucose and amino acids but lacking methionine. After 2 h of growth, the cells were washed and resuspended in M9 medium with 0.2% glucose. Expression of the Pf3 coat protein was induced with 1 mM IPTG for 10 min and pulselabeled with [^{35}S] methionine (10 μCi/ml) for 30 s. To generate spheroplasts, the pulse-labeled cells were collected by centrifugation at 4 °C and resuspended in 40% sucrose and 33 mM Tris-HCl (pH 8.0). Lysozyme (5 μg/ml) and 1 mM EDTA (pH 8.0) were added and kept on ice for 20 min. Where indicated, proteinase K was added (0.5 mg/ml) and incubated on ice for 1 h. Immunoprecipitation to Pf3, YidC, OmpA, and GroEL was performed as described previously (22). The samples were acid-precipitated and analyzed by SDS-PAGE.

Complementation with YidC-depleted Cells

E. coli MK6 cells bearing the respective plasmids were grown overnight in Luria broth (LB) medium with 0.2% arabinose and 25 μg/ml chloramphenicol and then diluted 1:100 into LB medium without arabinose. After 2h of growth the cells were serially diluted in 1:10 steps and spotted on LB plates containing 0.2% arabinose or 0.2% glucose, respectively. Where indicated, 1 mM IPTG was present. The plates were incubated overnight at 37 °C.

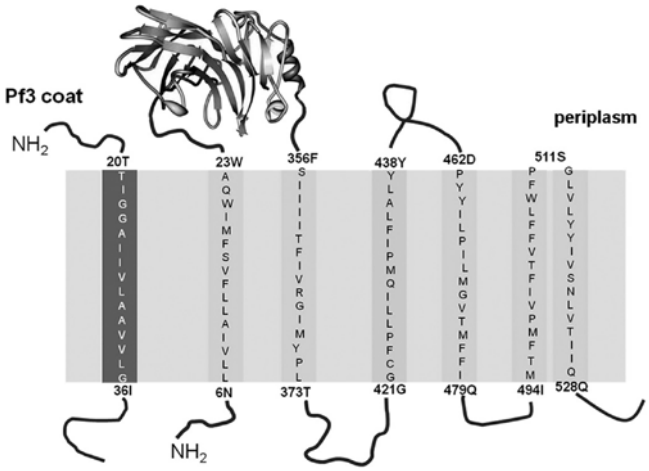

FIGURE 1 **Schematic representation of YidC and the Pf3 coat protein and their topology in the inner membrane of *E. coli*.** All indicated residues (single letter abbreviation) in the predicted membrane spanning segments (TM) of the Pf3 coat protein (left column) and YidC protein (right columns) were replaced by single cysteine residues. The gray area represents the membrane region.

RESULTS

The Pf3 Coat Protein Contacts YidC in the Center of TM1, TM3, TM4, and TM5

To follow the insertion process of the Pf3 coat protein, single cysteine mutants were generated in the Pf3 coat protein and in YidC (Figure 1). These were placed into each of the transmembrane regions covering all the membrane-spanning positions. In total, more than 100 single cysteine mutants were collected.

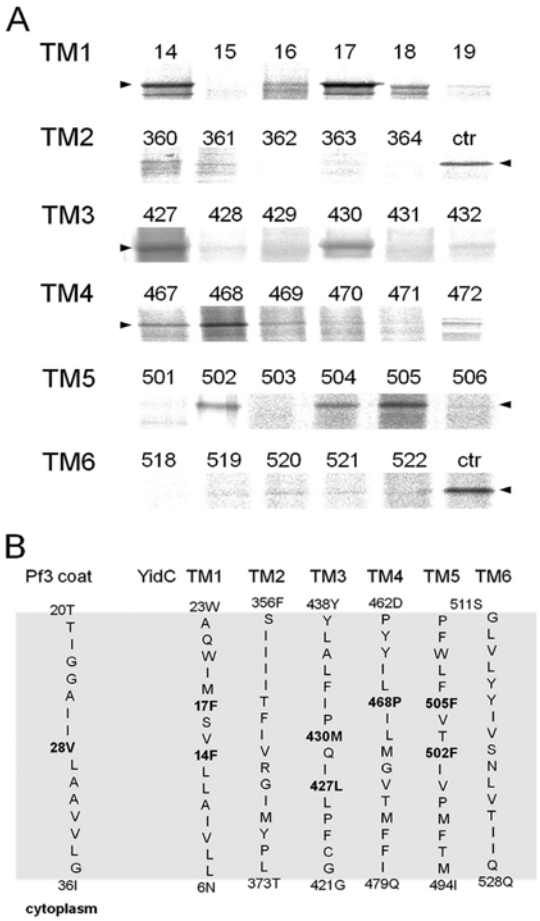

FIGURE 2 **Disulfide complexes of inserting Pf3-28C with YidC cysteine mutants in the center of TM1 to TM6. (*A)*** MK6 cells bearing the plasmid pMS-Pf3-28C and a second plasmid, pACYC, encoding the respective single cysteine YidC mutant, were grown in the presence of 0.2% glucose to deplete the cells from the chromosomally encoded YidC. The cells were induced with 1 mM IPTG for 10 min and pulse-labeled with [^{35}S] methionine for 30 s. The cells were put on ice and treated with 1mM copper phenanthroline. The cells were TCA-precipitated, immunoprecipitated with antibody to the Pf3 virus, and analyzed on non-reducing SDS-PAGE by phosphor imaging. For the control lanes, Pf3-28C and YidC430C (*ctr*) were coexpressed and treated as described above. The *arrowheads* depict the position of YidC-Pf3 complex. **(*B*)** Positions of the Pf3-28C contacts with YidC in the six transmembrane segments. The residues that provided stable disulfide cross-links when mutated to a cysteine residue are shown in *boldface*. The contacts were found in the center of TM1, 3, 4, and 5.

E. coli MK6 cells were transformed with a plasmid coding for one of the Pf3 coat protein single cysteine mutants and with a second plasmid coding for one of the YidC single cysteine mutants. The MK6 cells that have the chromosomal *yidC* under control of the araBAD promoter (18) were grown in glucose media to deplete the YidC wild-type and allow the plasmid-borne expression of YidC. All mutant proteins showed a comparable expression level. To map the contact sites during the insertion event, the two proteins were coexpressed in *E. coli* MK6 cells and pulse-labeled with [^{35}S] methionine for 30 s and treated with oxidant for 3 min. Then, the proteins were acid-precipitated and immunoprecipitated with an antibody to Pf3 coat protein. When the single cysteine residue was at position 28 of the Pf3 coat protein, which is in the center of the TM region, cross-linked YidC-Pf3 complexes were detected with certain cysteine mutants of YidC (Figure 2). These mutants had their cysteine residue in the center of TM1 at position 14 and 17, in TM3 at position 427 and 430, in TM4 at position 468, and in TM5 at positions 502 and 504/ 505. The neighboring positions showed significantly weaker or no cross-links. No strong contacts were detected in TM2 and TM6. To evaluate exactly the level of cross-linking, a positive control with cells coexpressing YidC 430C and Pf3–28C was added and loaded on the same gel.

The Pf3 coat protein contacts with YidC430 and 505 were analyzed in more detail (Figure 3). The pulse-labeled samples were immunoprecipitated either with Pf3 (Figure 3A, lanes 1–5) or with YidC antiserum (lanes 6–10) and analyzed by polyacrylamide electrophoresis, respectively. The contacts only occurred under oxidative conditions and when a cysteine is present on both protein partners (Figure 3, A and C). Under oxidative conditions, most of the Pf3 coat protein was present as dimers, but some monomers were also detectable. In addition, a significant proportion was found at about 65 kDa corresponding to a Pf3-YidC cross-link product. Clearly, the Pf3-YidC cross-link product was immunoprecipitated with both antibodies and migrated a little slower (Figure 3C, lanes 2-4) than the cysteine-less YidC on SDS-PAGE (lane 5). When the cysteine-less YidC was coexpressed, no YidC-cross-linked product was detectable (Figure 3A, lane 5). However, the formation of Pf3 coat protein dimers was unaffected. The contacts we observed with Pf3–28C were all at positions of the YidC protein that were predicted, on the basis of the hydrophobicity profile, to be in the center of the bilayer (Figure 2, lower panel), in TM1, 3, 4, and 5.

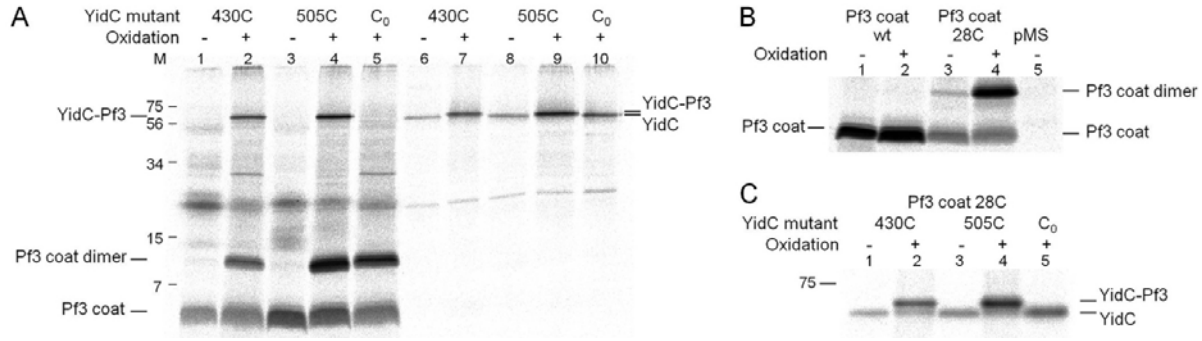

FIGURE 3 **Pf3-YidC disulfide complexes are recognized by antibodies to Pf3 and to YidC. (*A*)** Coexpression of Pf3-28C and YidC with a single cysteine at residue 430 (*lanes 1*, *2*, *6*, and *7*) and 505 (*lanes 3*, *4*, *8*, and *9*) were analyzed as described for Figure 2, except that in *lanes 1*, *3*, *6*, and *8* no copper phenanthroline was added. *Lanes 1-5* were immunoprecipitated with an antibody to Pf3. *Lanes 6-10* were immunoprecipitated with an antibody to YidC. As a control, the cysteine-less YidC mutant (*lanes 5* and *10*) was expressed. The Pf3 coat protein was found as a monomer, dimer, and cross-linked with YidC. Molecular weight markers are indicated in kDa at the left margin (*laneM*). **(*B*)** *E. coli MK6* cells expressing the wild-type Pf3 coat protein (*lanes 1* and *2*) and Pf3–28C (*lanes 3* and *4*) were pulse-labeled as described above and treated with copper phenanthroline (*lanes 2* and *4*). The empty vector is shown as a control (*lane 5*). **(*C*)** For better separation, *lanes 6-10* of Figure 3*A* were applied to a long PAGE.

Pf3 Contacts in the YidC Region Located in the Inner and Periplasmic Leaflet

We then tested which YidC residues were contacted by a Pf3 coat protein that had its single cysteine at position 33, predicted to localize in the inner leaflet of the membrane (Figure 4). MK6 cells bearing the plasmids for expressing a single cysteine mutant of YidC and a second plasmid expressing the Pf3 coat protein with a cysteine at position 33, respectively, were pulse-labeled for 30 s and analyzed for disulfide formation. YidC-Pf3 cross-link products were found to the YidC-TM1 (position 9), to TM3 (positions 424 and 427), and TM4 (position 476). Weak contacts were observed in TM3 at positions 425 and 428 that are next to the strong cross-linking signals and also in TM2 at positions 367 and 370. All these contacts are located in YidC at the predicted inner leaflet of the membrane bilayer (Figure 4, lower panel). However, no strong contacts were observed with TM5 and TM6 in the region of the inner leaflet on the basis of the comparison of the background signals with a control culture (coexpression of Pf3–28C and YidC-505C).

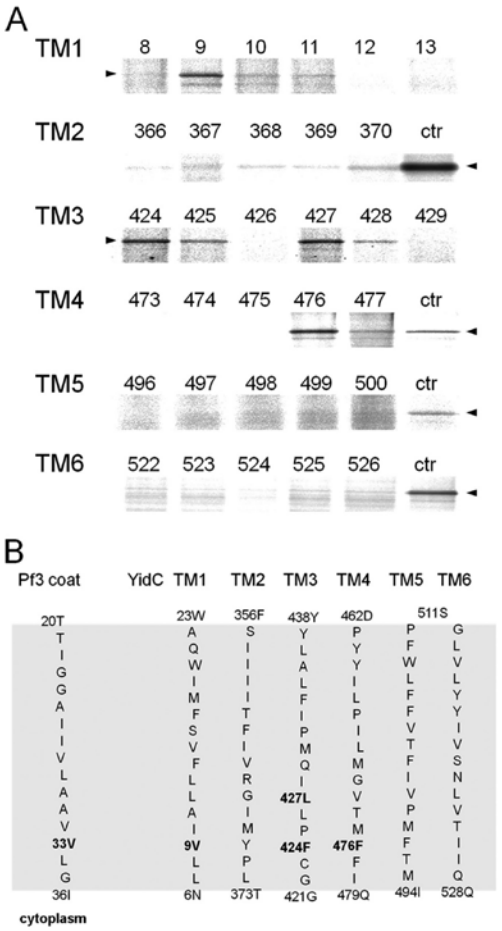

FIGURE 4 **Inserting Pf3-33C coat protein contacts YidC transmembrane residues located in the inner leaflet. (A)** *E. coli* MK6 cells bearing the plasmid pMS-Pf3-33C and a second plasmid encoding the respective single cysteine YidC mutant were grown and treated as described in the legend to Fig. 2. Cross-links were detected by immunoprecipitations with an antibody to Pf3. For the control lanes, Pf3-28C and YidC505C (ctr) were coexpressed and treated as described above. The arrowheads depict the position of the YidC-Pf3 complex. **(B)** Positions of the Pf3-33C contacts with YidC in the six transmembrane segments. The residues that provided stable disulfide cross-links when mutated to a cysteine residue are shown in boldface. The contacts were found in the inner half of TM1, 3, and 4.

Next, the YidC residues predicted to be located in the periplasmic leaflet of the transmembrane regions were investigated. When a Pf3 coat protein with a cysteine residue at position 24 was coexpressed, contacts were observed for YidC-TM3 at position 435 and for YidC-TM5 at 504 and 508 (Figure 5A). No major contacts were found with TM2 and TM4, as compared with the background. Weak interactions were detected with the YidC residue 18, 19 (in TM1) and 514 (in TM6). We noticed that in general, the YidC contacts to Pf3–24C were weaker than to Pf3–33C and Pf3–28C, presumably because the periplasmic contacts may occur for a shorter time.

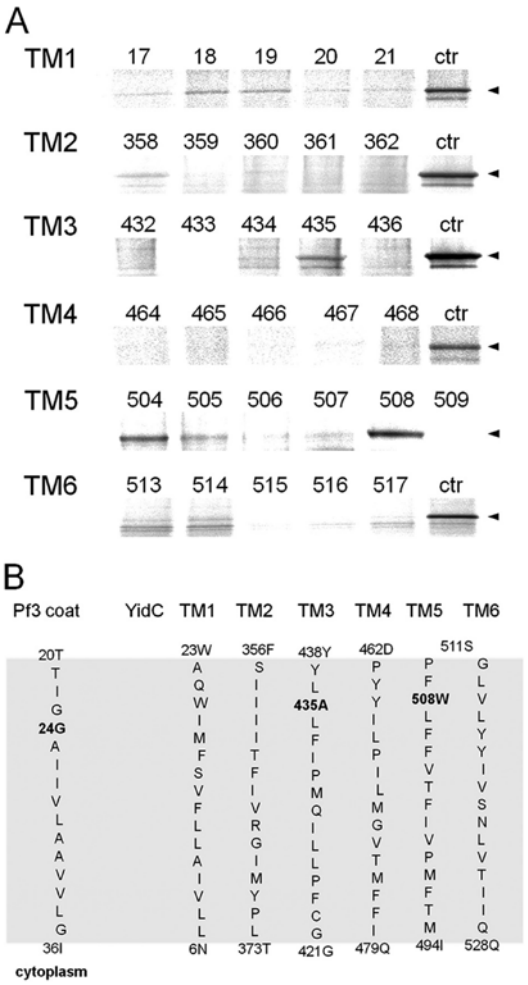

FIGURE 5 **Inserting Pf3-24C coat protein contacts YidC in the transmembrane residues of the outer leaflet. (A)** *E. coli* MK6 cells bearing the plasmid pMS-Pf3-24C and a second plasmid encoding the respective single cysteine YidC mutant were grown and treated as described in the legend to Fig. 2. Cross-links were detected by immunoprecipitations with an antibody to Pf3. For the control lanes, Pf3-28C and YidC505C (ctr) were coexpressed and treated as described above. The arrowheads depict the position of the YidC-Pf3 complex. **(B)** Positions of the Pf3-24C contacts with YidC in the six transmembrane segments. The residues that provided stable disulfide cross-links when mutated to a cysteine residue are shown in boldface. The contacts were found in the outer half of TM 3 and 5.

Taken together, we have observed 12 strong contact sites in TM1, 3, 4, and 5. They were often flanked by residues that also showed a contact to the substrate but, mostly, much weaker. This was the case for the flanking residues of residues 9, 17, 424, 427, 468, 477, and 505. The residues 504 and 505 gave about the same signal to Pf3–24C and Pf3–28C (Figs. 2 and 5), suggesting that both residues are equally contacting the substrate.

Contacts of Insertion-deficient Pf3 Coat Protein Mutants with YidC

To study the different stages of YidC-substrate interaction, experiments were performed with Pf3 coat protein mutants that are deficient for membrane insertion (24).

The Pf3-RR mutant has two arginines substituted for the amino acids at positions 17 and 18, whereas the Pf3-RS mutant has an arginine and a serine at these positions, respectively. A third mutant, Pf3-3R, is identical to Pf3-RR but has an additional arginine at position 7 replacing a glutamate. Single cysteines were introduced in all these mutants at position 24 to study their interaction with YidC and their insertion into the membrane. First, the membrane insertion of the mutants was studied in vivo by proteinase mapping (Fig. 6). *E. coli* BL-21 cells expressing the respective Pf3 coat protein mutant were grown in minimal medium lacking methionine to a density of 2 x 10^8 cells/ml. [^{35}S] methionine was added for 3 min, and the cells were analyzed for Pf3 coat protein insertion. Although the wild- type Pf3 coat was accessible to the protease added to the periplasmic side (lanes 2 and 4), the mutants Pf3-RS, Pf3-RR, and Pf3-3R were not digested by the protease demonstrating that they are inhibited for membrane insertion. For a control, the cytoplasmic protein GroEL and OmpA were immunoprecipitated, and we verified that the cells remained intact and the periplasm was accessible to the outside-added protease.

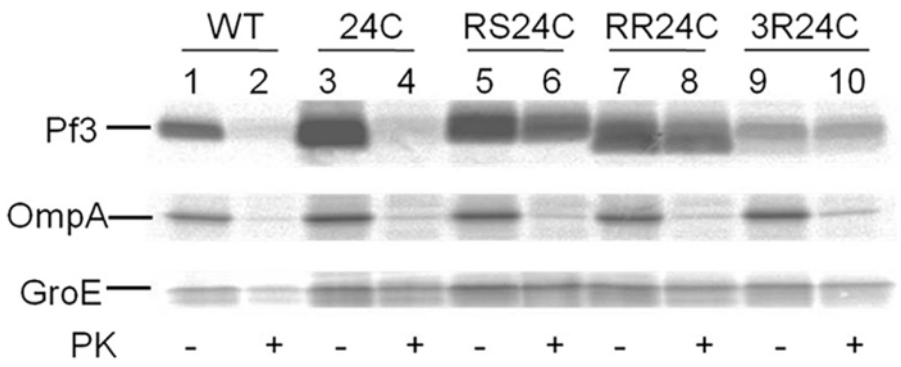

FIGURE 6 **Membrane insertion of Pf3-RS, Pf3-RR, and Pf3-3R is inhibited.** *E. coli* BL-21 cells expressing Pf3, Pf3-24C, Pf3-24C-RS, Pf3-24C-RR, or Pf3-24C-3R, respectively, were pulse-labeled for 3 min with [^{35}S] methionine. The outer membranes of the cells were opened by osmotic shock, and proteinase K (+ PK lanes) was added to the outside of the cells, and they were incubated on ice for 1 h. The samples were immunoprecipitated with antiserum to Pf3, GroEL, or OmpA, respectively, and analyzed by PAGE and phosphor imaging.

The Pf3-RR mutant was then coexpressed in *E. coli* MK6 with a single cysteine mutant of YidC and analyzed for contacts to YidC at the respective positions (Fig. 7). When the cysteine at position 24 in the Pf3 wild-type (Fig. 7A) was compared with Pf3-RR-24C (B), the contacts to the YidC residues at 435 and 508 were substantially reduced in the insertion-deficient Pf3-RR mutant. These residues are in the periplasmic membrane

portion of YidC. Similarly, the residues in the center of the membrane at 430, 468, and 505 were not or only weakly cross-linked with Pf3-RR with a cysteine at position 28 (Fig. 7D). However, the YidC residues 424, 427, and 476 located in the inner leaflet were not inhibited for contacting Pf3-RR-33C (compare Fig. 7, E and F). The Pf3–3R mutant containing three arginine residues in the amino terminal domain was also tested for YidC interaction (Fig. 8). When this Pf3-3R mutant had a cysteine at position 24, 28, or 33, it was inhibited to interact with the residues 508, 505, and 476 of YidC located in the outer, central, and inner leaflet, respectively (Fig. 8A). When the samples were immunoprecipitated with antibodies to YidC, the shift of the band caused by a bound Pf3 protein was not observed when the 3R mutations were present in Pf3 (Fig. 8B), indicating that this Pf3 mutant is unable to interact with the YidC insertase.

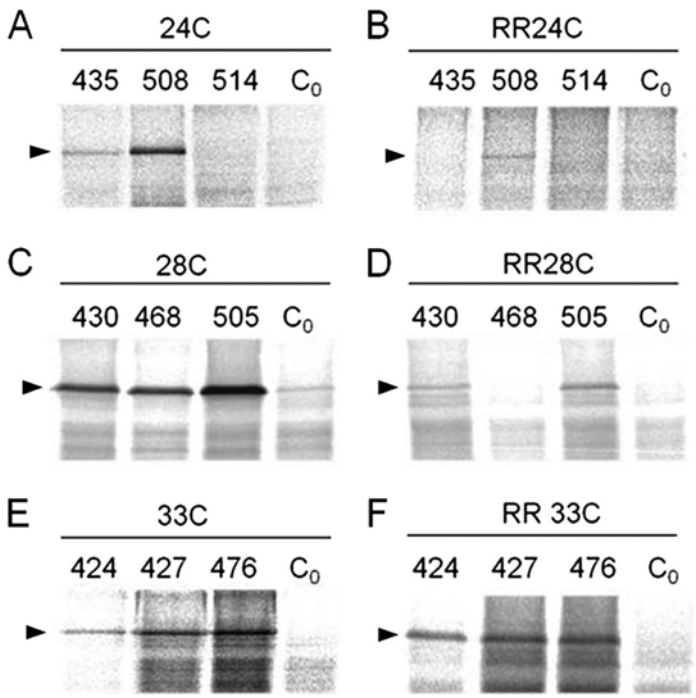

FIGURE 7 **Pf3-RR is blocked for contacting the YidC residues in the outer leaflet.** *E. coli* MK6 cells expressing Pf3-24C **(A)**, Pf3-24C-RR **(B)**, Pf3-28C **(C)**, Pf3-28C-RR **(D)**, Pf3-33C **(E)**, or Pf3-33C-RR **(F)** were coexpressed with the respective YidC mutants to analyze close contacts by disulfide crosslinking. The cells were labeled with [^{35}S] methionine for 30 s and immunoprecipitated with antiserum to Pf3. As a control, the cysteine-less YidC (Co) was coexpressed. The samples were analyzed by PAGE and phosphor imaging.

YidC Function Is Affected When Multiple Contact Sites Are Mutated

A further way to show that the contacting residues are important for function is to substitute all these residues into alanines or serines. We rationalized that for the YidC residues that are involved in substrate interactions are most likely not involved in helix-helix interactions important for the structure of the YidC protein. Hence, the structure of YidC should not be affected when only the contacting residues are mutated. However, it is expected that the substrate interaction is disturbed when the contacting YidC residues are changed to alanines and to serines, which are less hydrophobic than the corresponding residue in the wild-type. YidC function can be assessed in the MK6 strain where the chromosomal YidC is under control of the arabinose promoter. When YidC is depleted under glucose conditions, growth is inhibited unless a plasmid with a functional yidC gene is present and induced. In a mutant where the nine major contacting residues were substituted to serines, termed YidC-9S, YidC function was affected, and the plasmid-derived copy did no longer complement the chromosomally encoded gene (Fig. 9, right panel).

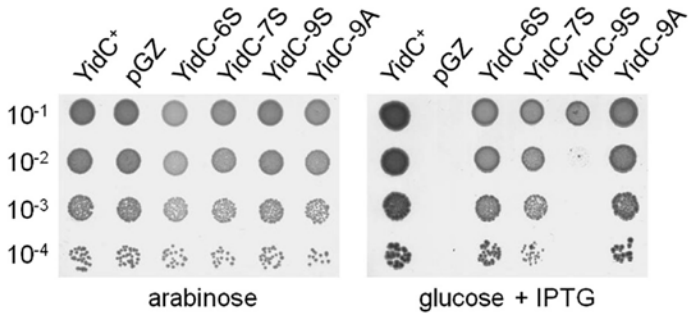

FIGURE 8 **Cumulative mutations in the substrate contacting residues of YidC inhibit its function.** The Pf3 contacting residues at 424, 427, 430, 435, 468, 476, 502, 505, and 508 were substituted to Ala (YidC-9A) and to Ser (YidC-9S) and expressed in MK6 under YidC-depleted conditions (in the presence of glucose, *right panel*) or non-depleted conditions (in the presence of arabinose, *left panel*). A mutant that has the contacting residues in the central and periplasmic leaflet (YidC-6S; 430, 435, 468, 502, 505, 508) and a mutant in the central and cytoplasmic positions mutated (YidC-7S; 424, 427, 430, 468, 476, 502, 505) were tested. 1 mM IPTG was added to induce the plasmid-directed synthesis of each YidC mutant. Serial dilutions of the cultures were spotted and incubated over night at 37 °C. For a control, cells transformed with the empty plasmid (*pGZ*) and cells encoding the wild-type YidC (*YidC*+) are shown.

In contrast, the YidC mutant with alanines at these positions still complemented the chromosomally encoded gene. Interestingly, cell growth was still observed when only the residues located at the central and periplasmic positions in the transmembrane segments were changed to serines (YidC-6S). Likewise, when the residue at the central

and cytoplasmic positions was changed to serine (YidC-7S), growth was not affected. Taken together, we conclude that YidC function is only distorted when multiple contacting residues along the entire membrane region were changed.

DISCUSSION

The membrane insertase YidC catalyzes the insertion of the Pf3 coat protein into the inner membrane of E. coli (11). During this process, the Pf3 protein binds to YidC mainly by hydrophobic interactions (9) and induces a conformational change within YidC (10, 25). As a result, the Pf3 coat protein adopts a transmembrane conformation, exposing its N-terminal domain to the periplasm. Although the substrate binding step most likely involves the cytoplasmic residues of YidC, insertion of the TM region and translocation of the N-terminal region are likely to require other contacting residues of YidC. The molecular interactions of newly synthesized Pf3 coat protein were analyzed during 30 s after the onset of its synthesis. The data presented here show that multiple YidC-substrate contacts are observed involving the participation of most of the transmembrane segments of YidC. Only strong disulfide signals were considered. The weak signals were mostly observed at positions flanking the strong signals. Weak signals at other positions (e.g. at 358, 360, 370, and 514) may arise from a structural flexibility of YidC. Although TM3 showed contacts all across the predicted membrane-spanning α-helix, TM5 has three positions (502, 505, and 508), located in the central and outer leaflet, that make a contact with the newly expressed Pf3 coat protein. These contacts reveal a first three-dimensional picture of how the substrate is possibly binding YidC (Fig. 10). In the cytoplasmic leaflet, the substrate contacts TM1, 3, and 4 of YidC, whereas in the periplasmic leaflet, TM3 and 5 are contacted. This suggests that the residues at positions 424, 427, and 476 are primarily involved in the first substrate binding step, whereas residues 430, 435, 468, 502, 505, and 508 are required for translocation. The data that we have obtained with the substrate mutant Pf3-RR that is defective for membrane insertion are in support of this model. The insertion-deficient mutant RR-Pf3 was contacting 424, 427, and 476 in the cytoplasmic leaflet. However, the contacts to the central and periplasmic residues 430, 435, 468, 505 and 508 were substantially reduced (Fig. 7).

Intriguingly, the residues of YidC that are most conserved among the bacterial homologues are present in TM2, TM3, and in the C1 loop (12). Also, the periplasmic region of TM5 shows a high degree of homology (26). In these conserved TM regions of YidC we found seven major contacting residues. Surprisingly, several contacts were found within TM1, which is less conserved and is missing in Gram-positive bacteria as well as in the mitochondrial and thylakoidal homologues. This suggests that TM1 contributes to the YidC function, although it is not essential in the YidC family of proteins. The importance of the other TM regions for the function of YidC has been implicated from a number of site-directed mutants (17). The deletion of these TM regions inactivates the protein. Single residue substitutions had shown that only a few residues within the transmembrane regions are sensitive. Most of these locate in TM3, corroborating its central role for the insertase activity (17). Interestingly, the critical residues are not at the exact positions where our substrate contacts are localized but adjacent to them. Most likely, these residues are not involved in substrate interaction but rather in helix-helix contacts within YidC. One such contact was proposed for residue 423 in TM3 with TM2 (27). When the cysteine at 423 was substituted for an arginine, a cold-sensitive phenotype was observed. Second-site suppressors identified residue T362E forming a possible charge pair with Arg-423, suggesting thatTM2andTM3are close together. A further suppressor mutation of Arg-423 within TM3 was found at L426E in the same TM, indicating that residues 362, 423, and 426 are involved in helix-helix interaction and not in substrate binding. In accordance with this, we did not find Pf3-YidC contacts at 362, 423, or 426.

A previous study (19) had also identified TM3 of YidC as the substrate contact site. However, different residues were found to form disulfide contacts to the in vitro synthesized substrates, possibly because the cysteine mutants at positions 427 and 428 were not available. The Sec-dependent FtsQ protein was found to contact residues 425 and 426. With residue 425, we also found a weak interaction with Pf3, but residue 426 did not form a disulfide with Pf3. Another difference between that study and ours is that they had a nascent chain-arrested ribosome interacting with YidC in membrane vesicles in vitro. It is possible that the nascent chain could bind to the outside of YidC. Our experimental approach was in vivo, and to detect the formed disulfides we looked shortly (30-s pulse) after the addition of [^{35}S] methionine. In this time span we expected to observe the naturally occurring contacts. In our results, the transmembrane segment

TM3 showed four contacting residues at 424, 427, 430, and 435, corroborating that it is the most important YidC helix involved in substrate binding. When the residues are projected as a helical wheel, the contacting residues are on the same helical face (Fig. 10B). A similar result was obtained for TM1 and TM5, where more than one contact was observed. All substrate-contacting residues are hydrophobic. In particular, the extremely hydrophobic phenylalanine was found in six different contact sites. Because these phenylalanine residues are located in four different transmembrane helices, they together may constitute a stacking-like arrangement over the entire membrane-spanning region. In conclusion, the substrate-interacting surface of YidC is hydrophobic across the entire membrane span. We propose that the contacting residues form a hydrophobic platform that supports the transmembrane alignment of an incoming α-helix of the substrate. This proposed mechanism differs from the one of SecY, which is mostly hydrophilic with a small hydrophobic pore ring in its center (28).

One important question is whether the observed contacting residues are indeed of functional importance for membrane insertion. To answer this question, we studied Pf3 mutants that were deficient for membrane insertion. These mutants should fail to contact YidC at least at the periplasmic positions. The Pf3-RR mutant has an alanine residue at position 17 and an aspartic acid residue at position 18 substituted with arginines. Pf3-RR was not found to contact YidC at the periplasmic and central positions. For the Pf3-3R mutant, which had the aspartic acid at position 7 substituted to an arginine, in addition to the two arginines at 17 and 18, showed no contacts to YidC, not even to the cytoplasmic residue at 476 (Fig. 8). These data suggest that the Pf3-RR mutant protein still binds to the cytoplasmic side of YidC but cannot reach out to contact the more periplasmically located residues of YidC. Therefore, the translocation process of Pf3-RR across the membrane was perturbed, and the Pf3-3R mutant appeared to be blocked for both binding and translocation.

Previously, the TM regions of YidC had been analyzed by serine scanning, and thereby most of the contacting residues had been mutated to serine individually (17). All of these mutants turned out to be functional. However, in our study we found that when we combined the serine mutations of most major contacting sites, YidC function was severely affected (Fig. 9).

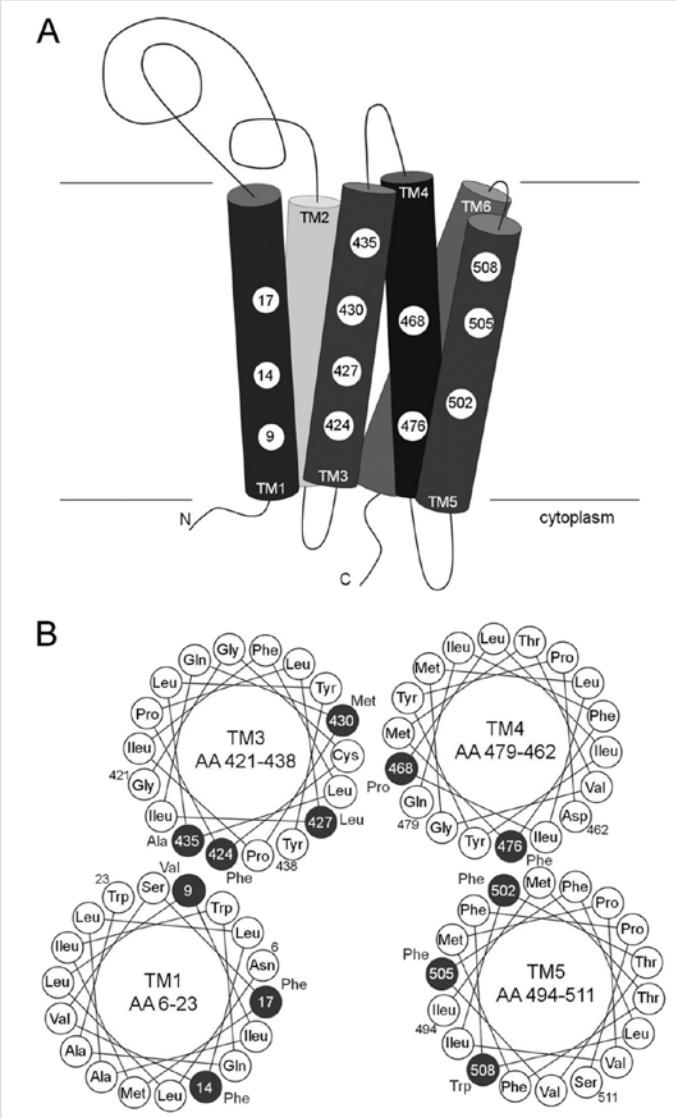

FIGURE 10 **Model of the YidC hydrophobic protein binding platform. (A)** The major contact sites in TM1, 3, 4, and 5 are depicted by a numbered circle referring to the amino acid residue position. **(B)** Helical wheel projection to illustrate the substrate contact sites of the YidC TM regions. The major contact sites are highlighted as dark circles with the respective position. Both ends of each TM segment are labeled with the position number outside the circle. Because TM2 and TM6 showed no strong contacts, they are not included in the model.

Taken together, our data show that YidC contacts its substrate Pf3 at multiple sites within the transmembrane domain (Fig. 10). This is in line with the proposal that YidC provides a hydrophobic platform supporting membrane insertion of proteins (29). The contacts are required for the insertase activity because the Pf3 cysteine mutants that are defective for membrane insertion were impaired to contact YidC.

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CONTRUBTION TO THIS MANUSCRIPT

C.K. and A.K. designed the experiments. C.K. performed all experiments. C.K and A.K. prepared the figures. C.K. and A.K. wrote the manuscript. A.K. supervised and directed the research.

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Chapter 4

Summary

YidC of *Escherichia* coli belongs to the evolutionarily conserved proteins of the Oxa1/YidC/Alb3 insertase family. The transmembrane regions of the core domain, comprising of TM2-6, are the most conserved parts among the homologs and are crucial for the function as a membrane insertase. This is particularly true for the TM2, TM3 and TM5 (KUHN et al., 2003; KIEFER & KUHN, 2007). In bacteria, YidC acts as an independently working membrane insertase and, as well, in cooperation with the Sec translocon for the biogenesis of various membrane proteins. YidC is required for the biogenesis of respiratory complexes, ATP synthase and for example the mechanosensitive channel protein MscL. Also, the coat proteins of filamentous phage Pf3 and M13 require YidC for membrane insertion. The best studied substrate is the Pf3 coat protein of phage Pf3 infecting *Pseudomonas aeruginosa* – i.e. a small protein of 44 amino acids in length.

In the context of this thesis, the YidC-dependent biogenesis of Pf3 coat was analyzed to gain better insight into the entire insertion process. In doing so, a set of more than 100 single cysteine mutants in distinct domains of YidC and Pf3 coat were generated. To study the insertion of Pf3 coat under physiological conditions, an *in vivo* cross-linking assay was established for capturing YidC-Pf3 interactions within a short period of time after the onset of synthesis (1 minute) using ^{35}S-Met pulse-labelling methods.

YidC binds inserting Pf3 coat protein in distinct regions of the highly conserved TM domains involving four of the six TM helices. It was verified that TM3 is indispensable for the function of YidC since four contacting residues were found in this TM helix. A helical wheel projection of substrate binding helices reveals the localization of the contacting residues of each TM segment on one helical face. This implies a helix arrangement of the transmembrane core domain which enables binding of inserting substrate proteins and interactions with transmembrane domains over the entire membrane-spanning part of YidC. The serial mutation of nine from twelve contacting residues, which are strongly hydrophobic in most cases, to serines impaired the function of YidC, whereas the single mutations had no effect.

Additionally, the insertion process of translocation deficient Pf3 coat mutants was analyzed for intermediate states of the insertion process. It has been shown that the insertion deficient Pf3 coat mutants are inhibited at a late step of membrane insertion, i.e. forming the YidC contacts in the periplasmic leaflet.

Based on this work, further studies confirmed that the identified substrate contacting regions of YidC play a key role in YidC-mediated insertion. The mechanosensitive channel protein MscL, M13 procoat, nascent F_oc and the polytopic membrane protein LacY contact YidC at exactly the same positions (NEUGEBAUER et al., 2012; SPANN & KUHN, unpublished results; WICKLES et al., 2014; ZHU et al., 2013b).

Zusammenfassung

YidC von *Escherichia coli* gehört zu den evolutionär konservierten Proteine der Oxa1/ YidC/Alb3 Insertase Familie. Die Transmembranbereiche (TM) der Kerndomäne, die die TM2-6 umfasst, sind die konserviertesten Bereiche unter den Homologen und sind entscheidend für ihre Funktion als Membraninsertase. Dies gilt insbesondere für die TM2, TM3 und TM5 (KUHN et al., 2003; KIEFER & KUHN, 2007). In Prokaryonten wirkt YidC als unabhängig arbeitende Membraninsertase und in Zusammenarbeit mit dem Sec-Translokon in Prozessen der Biogenese von Membranproteinen. YidC wird für die Assemblierung von Komplexen der Atmungskette, der ATP-Synthase und beispielsweise für die Biogenese des mechanosensitiven Kanalproteins MscL benötigt. Außerdem benötigen die Hüllproteine der fadenförmigen Phagen Pf3 und M13 YidC für die Membraninsertion. Das am besten untersuchte Substrat ist das Hüllprotein des Phagen Pf3, der *Pseudomonas aeruginosa* infiziert – es ist ein kleines Protein mit einer Länge von 44 Aminosäuren.

Im Rahmen dieser Arbeit wurde die YidC-abhängige Biogenese des Pf3 coat Proteins untersucht, um einen besseren Einblick in den Mechanismus des gesamten Insertionsprozesses zu gewinnen. Dabei wurden mehr als 100 Einzelcysteinmutanten in verschiedenen Domänen des YidC und Pf3 coat Proteins erzeugt. Für die Untersuchung der Pf3 coat Insertion unter physiologischen Bedingungen wurde eine *in vivo* Quervernetzungsmethode etabliert, die es ermöglichte YidC-Pf3 Interaktionen innerhalb der Dauer von einer Minute nach Synthesebeginn in „^{35}S-Met-Pulse-labelling“ Experimenten zu untersuchen.

YidC bindet inserierendes Pf3 coat Protein in unterschiedlichen Bereichen der hochkonservierten Transmembrandomänen. Die Bindung erfolgt an vier der sechs Transmembrandomänen. Die Wichtigkeit der TM3 für die Funktion von YidC wurde bestätigt. In dieser TM wurden vier substratbindende Aminosäuren identifiziert. Die „helical-wheel projection“ der substratbindenden Helices zeigte, dass die kontaktierenden Aminosäuren jeder Transmembrandomäne auf einer Seite der jeweiligen Helix lokalisieren. Dies impliziert eine Anordnung der membranständigen Kerndomäne, die eine Bindung des inserierenden Substratproteins und Wechselwirkungen zwischen Transmembrandomänen über den gesamten membranspannenden Teil von YidC

ermöglicht. Die gleichzeitige Mutation von neun der zwölf kontaktierenden, meist stark hydrophoben Aminosäuren zu der leicht polaren Aminosäure Serin beeinträchtigte die Funktion von YidC, während die Einzelmutationen keinen Effekt zeigten.

Zusätzlich wurde der Insertionsprozess von translokationsinhibierten Pf3 coat Mutanten untersucht. Es wurde gezeigt, dass die Bindung von insertionsdefizienten Pf3 coat Proteinen an YidC in einem späteren Schritt der Membraninsertion gehemmt ist.

Auf der Grundlage dieser Arbeit, konnten weitere unabhängige Studien bestätigen, dass die identifizierten substratbindenden Bereiche von YidC eine universelle Funktion in der YidC-abhängigen Insertion besitzen. Es wurde gezeigt, dass das mechanosensitive Protein MscL, M13 procoat, naszierendes F_oc Protein und das polytope Membranprotein LacY YidC an genau den gleichen Positionen kontaktiert (NEUGEBAUER et al. 2012; SPANN & KUHN, unveröffentlichte Ergebnisse; WICKLES et al., 2014; ZHU et al., 2013).

Concluding remarks and outlook

The recent publication of the crystal structures of *Bacillus halodurans* YidC2 and *Escherichia coli* YidC is a breakthrough in the field of YidC-mediated insertion (KUMAZAKI et al., 2014a; KUMAZAKI et al., 2014b). Most likely, the hydrophilic groove and the flexibility of the C1 region and the attached TM3 represent the fundamental structural features of YidC, necessary to promote membrane insertion of substrate proteins. Both YidC structures suggest an insertion mechanism in which newly synthesized substrates are guided to the highly flexible C1 region as a first step (Figure 1.21A). The polar region of the inserting substrate protein is then attracted by residues coating the interior of the hydrophilic groove which is open towards the cytoplasm and the membrane (Figure 1.21B). By this means, the polar region of the inserting substrate is allowed to penetrate into the middle of the lipid bilayer within the hydrophilic groove during initial events in the insertion process. Consequently, YidC seems to lower the energy barrier which has to overcome for the partial integration of substrate TM domains into the membrane. A prominent role in this mechanism was designated to the conserved single arginine residue which is located in the center of the hydrophilic groove in both structures.

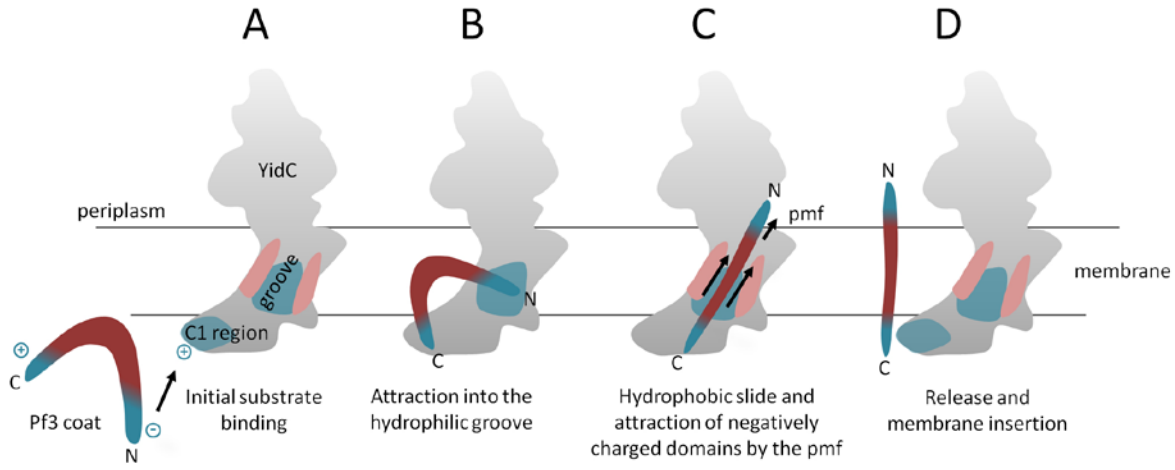

FIGURE 1.22 **Proposed stages of YidC mediated membrane insertion.** The model is based on the combination of data from the recently published crystal structure of *Escherichia coli* YidC and data from the present thesis. The transmembrane domain of Pf3 coat protein is colored in red. Flanking hydrophilic regions are colored in blue. The hydrophilic cavity of YidC is colored in blue as well as the positively charged C1 region. Hydrophobic faces of transmembrane helices flanking the hydrophilic cavity are shown as parallel stripes on both sides of the cavity and colored in salmon. **(A-D)** Four proposed stages of YidC mediated membrane insertion (see text for details).

However, this seems to be only true for SpoIIIJ of *B. subtilis* and *Streptococcus mutans* YidC2 but not for *E. coli* YidC since there a substitution mutant to alanine was fully functional (KUMAZAKI et al., 2014a; KUMAZAKI et al., 2014b; CHEN et al., 2014). In the next step of insertion, the polar substrate region is released from the hydrophilic groove to transit the membrane core region (Figure 1.21C). One possibility would be that the membrane potential attracts negatively charged residues located in regions which are destined to reside in the periplasm. This mechanism might be supported by the hydrophobic interaction of the TM regions of YidC and the TM regions of the substrate.

In the present thesis, strong evidences for the importance of hydrophobic interactions between YidC and inserting substrates in order to facilitate the final insertion of the substrates transmembrane regions were gained. Multiple substrate contacting residues were found in the highly conserved transmembrane segments TM3 and TM5 which constitute the border TM helices of the hydrophilic groove opening (KUMAZAKI et al., 2014b; KLENNER & KUHN, 2012). The crystal structure clearly confirms that the substrate contacting residues are located mainly on one helical face of the respective TM domain (Figure 1.22).

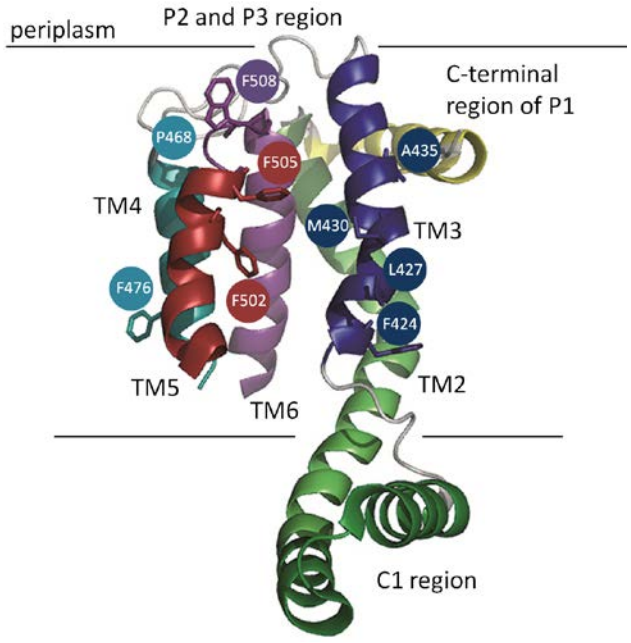

FIGURE 1.22 **Localization of substrate-contacting residues in the vicinity of the hydrophilic groove.** Cross section of the membrane - looking into the hydrophilic groove. The residues which were successfully cross-linked to inserting Pf3 coat protein are highlighted by numbers in the crystal structure (PDB entry 3WVF). The residues are indicated by sticks and labeled in colored circles.

Since most of the substrate contacting residues are strong hydrophobic (KLENNER & KUHN, 2012) it thus stands to reason that the hydrophobic parts of TM helices flanking the cavity enable a "hydrophobic slide" of substrate TM segments along YidC during translocation of the hydrophilic parts across the periplasmic leaflet (DALBEY & KUHN, 2014) (Figure 1.21C). Finally, the substrate adopts its transmembrane conformation and is released from YidC (Figure 1.21D).

Thus, it is conceivable that hydrophobic interactions between YidC TM segments facing the core region of the membrane and substrate TM segments lead to the final insertion into the bilayer. Interestingly, the YidC-independent proteins FtsQ and LacY contact similar regions of YidC during membrane biogenesis. This suggests that YidC TM3 and TM5 helices are important for the proposed activity of YidC as a molecular chaperon.

Based on the present data of the detailed cysteine cross-linking studies and the crystal structure of *E. coli* YidC further experiments will elucidate YidC mediated insertion in even more detail. Future questions will address how YidC handles different substrates and how the insertase cooperates with the Sec translocon. Especially the purification and crystallization of insertion intermediates of different substrates bound to YidC could be helpful to fully elucidate distinct steps in the insertion mechanism.

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Publications

Christian Klenner, Jijun Yuan, Ross E. Dalbey and Andreas Kuhn (2008) The Pf3 coat protein contacts TM1 and TM3 of YidC during membrane biogenesis. *FEBS Lett* ***582****, 3967-72*

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1. Bei der eingereichten Dissertation zum Thema

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