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Minireview

MINIREVIEW – Physiology & Biochemistry

Co-translational protein targeting in bacteria

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One sentence summary: A comprehensive review of protein targeting strategies in bacteria with emphasis on the co-translational targeting by the signal recognition particle.

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ABSTRACT

About 30% of all bacterial proteins execute their function outside of the cytosol and have to be transported into or across the cytoplasmic membrane. Bacteria use multiple protein transport systems in parallel, but the majority of proteins engage two distinct targeting systems. One is the co-translational targeting by two universally conserved GTPases, the signal recognition particle (SRP) and its receptor FtsY, which deliver inner membrane proteins to either the SecYEG translocon or the YidC insertase for membrane insertion. The other targeting system depends on the ATPase SecA, which targets secretory proteins, i.e. periplasmic and outer membrane proteins, to SecYEG for their subsequent ATP-dependent translocation. While SRP selects its substrates already very early during their synthesis, the recognition of secretory proteins by SecA is believed to occur primarily after translation termination, i.e. post-translationally. In this review we highlight recent progress on how SRP recognizes its substrates at the ribosome and how the fidelity of the targeting reaction to SecYEG is maintained. We furthermore discuss similarities and differences in the SRP-dependent targeting to either SecYEG or YidC and summarize recent results that suggest that some membrane proteins are co-translationally targeted by SecA.

Keywords: signal recognition particle; FtsY; SecYEG translocon; YidC; protein targeting; SecA

INTRODUCTION

The remarkable metabolic plasticity of bacterial cells depends on the spatial and temporal coordination of multiple molecular processes that allow bacteria to rapidly adapt to their constantly changing environment. Coordination of protein transport processes is a crucial part of this adaptation. It depends on a variety of sophisticated protein delivery systems that maintain cellular integrity by routing proteins from the cytosol to their correct destination within the cytoplasmic membrane, the periplasmic space, the outer membrane or the extracellular space. These protein delivery systems are generally classified as co-translational, i.e. when protein transport is coupled to protein synthesis or as post-translational, i.e. when transport is disconnected from translation (Mori and Ito 2001; Driessen and Nouwen 2008; Akopian *et al.* 2013; Kudva *et al.* 2013; Rapoport, Li and Park 2017). The intrinsic advantage of co-translational targeting is the reduced risk of protein aggregation in the cytosol, and bacteria use this system primarily for aggregation-prone membrane proteins (Kuhn *et al.* 2014). A disadvantage is that transport is limited by the slow translation rate (Rodnina and Wintermeyer 2016) and as a consequence, a large portion of the anyway limited number of protein transport channels is occupied by the translating ribosome. This is different for post-translational targeting systems, which maintain substrates in a

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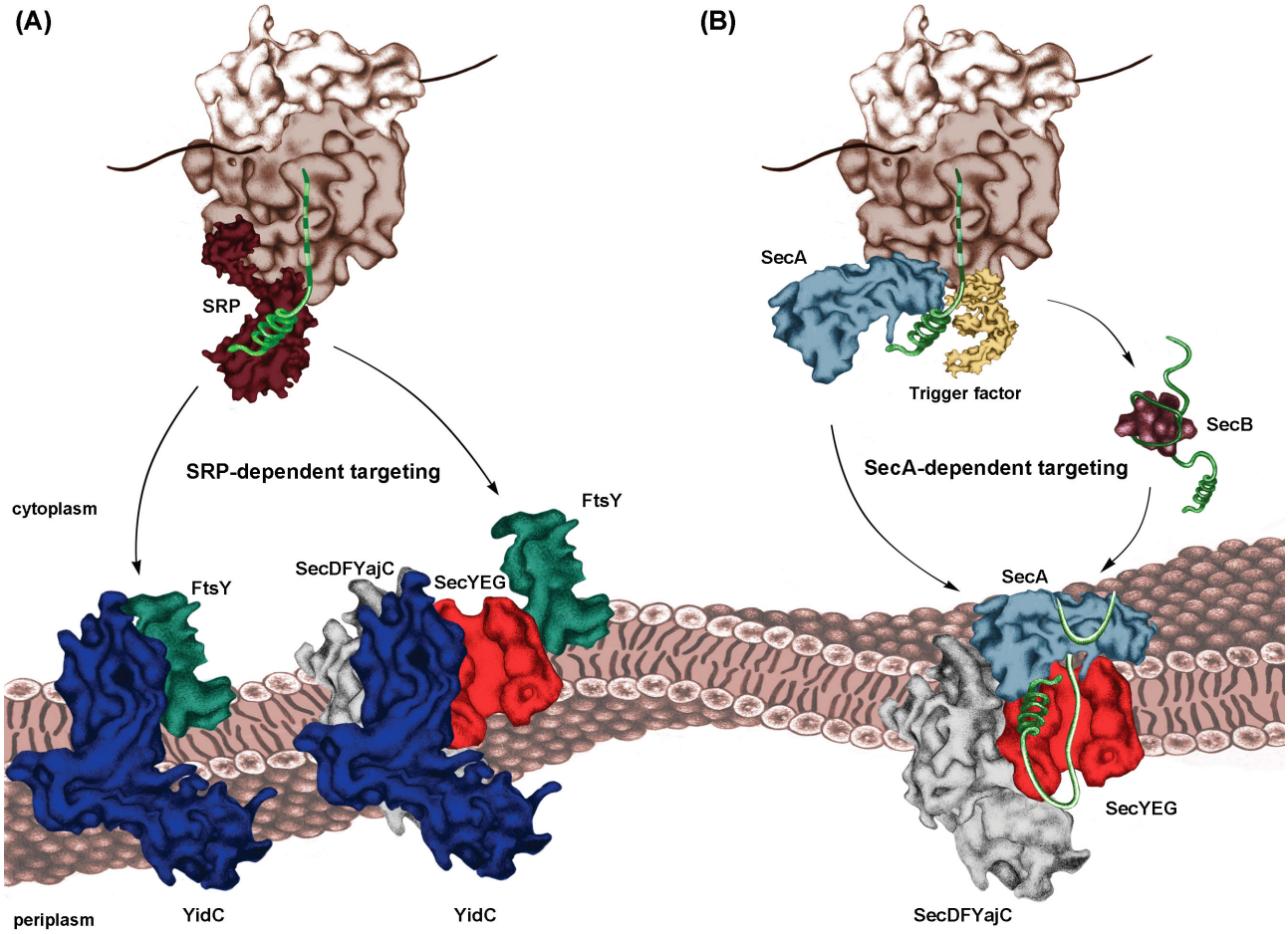


Figure 1. Co- and post-translational protein targeting to the SecYEG translocon and to the YidC insertase in bacteria. Bacteria use distinct pathways for the transport of inner membrane proteins or secretory proteins, i.e. periplasmic and outer membrane proteins. (**A**) Targeting of inner membrane proteins is initiated by the co-translational binding of SRP (dark red) to ribosomes (brown) translating a membrane protein (light green, the dotted part indicates the portion of the nascent chain that is still inside the ribosomal tunnel). The SRP–ribosome-nascent chain complex is then targeted to the SR FtsY (dark green), which is bound to the membrane-integral SecYEG translocon (red). Conformational rearrangements within the quaternary SecYEG–FtsY–SRP–RNC complex (not shown), allow docking of the RNC onto the SecYEG translocon and GTP hydrolysis by SRP and FtsY. This releases SRP back into the cytosol, while FtsY stays in contact with either SecYEG or lipids. Ongoing translation and lipid partioning drives the insertion of TMs into the membrane. The heterotrimeric SecYEG translocon forms a protein conducting channel and associates at least transiently with additional proteins, like YidC (blue) or the SecDFYajC complex (gray). YidC assists the release of membrane proteins from the channel, while the SecDFYajC complex is thought to assist proton-motive-force-dependent steps during protein transport. SRP can deliver RNCs of membrane proteins also to YidC, but insertion via YidC is limited to membrane proteins lacking large periplasmic loops. Although FtsY can also bind to YidC, it is currently unknown whether SRP-dependent targeting to YidC follows the same principles as targeting to SecYEG. (**B**) Secretory proteins are first contacted by the ribosome-associated chaperone trigger factor (yellow). After their release from the ribosome they are either directly bound by the SecYEG-bound ATPase SecA (light blue), which translocates the secretory proteins post-translationally in ATP-dependent steps through the SecYEG channel. Alternatively, some secretory proteins are first bound by the secretion-specific chaperone SecB (dark brown) and only then get into contact with the SecYEG-bound SecA. During transport, the signal sequence of secretory proteins is cleaved off by signal peptidases. SecDFYajC (gray) can also associate with the translocating SecYEG translocon. Recent data demonstrate that SecA can also interact with ribosomes and RNCs, suggesting a co-translational recognition event by SecA. To which extent SecA is engaged in ribosome binding and co-translational recognition/targeting requires further analyses.

transport-competent conformation until a transport channel is available (Tsirigotaki *et al.* 2017). Typical substrates here are less hydrophobic periplasmic and outer membrane proteins, collectively called secretory proteins. As transport is separated from translation, transport is generally faster but requires an additional driving force, which is provided by ATP hydrolysis and the proton motive force (Karamanou *et al.* 1999; Tomkiewicz *et al.* 2006; Knyazev *et al.* 2018). Accessory proteins of the SecYEG translocon, like the SecDFYajC complex probably further aid the translocation process (Tsukazaki *et al.* 2011; Fig. 1).

The best studied system for co-translational targeting depends on the signal recognition particle (SRP) and its receptor, termed FtsY in bacteria. The SRP system was initially identified in eukaryotic cells for protein transport into the endoplasmic reticulum and only later homologues in bacteria and archaea were discovered (Bernstein *et al.* 1989; Romisch *et al.* 1989; Pohlschroder *et al.* 2005; Yuan *et al.* 2010). The contribution of the bacterial SRP system to protein transport was initially controversially discussed (Johnson, Murphy and Beckwith 1992) and only after the development of purified systems the importance of the bacterial SRP for membrane protein targeting was established (Luirink *et al.* 1994; Powers and Walter 1997; Valent *et al.* 1998; Koch *et al.* 1999; Table 1). The intrinsic feature of SRP to bind to ribosomes in prokaryotes and eukaryotes (Pool *et al.* 2002; Gu *et al.* 2003; Halic *et al.* 2004; Schaffitzel *et al.* 2006) allows it to scan translating ribosomes for correct substrates.

SRP then initiates targeting of ribosome-associated nascent chains (RNCs) to the membrane-bound FtsY and ultimately to either the SecYEG translocon (Valent *et al.* 1998; Koch *et al.* 1999) or the YidC insertase (Facey *et al.* 2007; Welte *et al.* 2012) for co-translational insertion into the membrane (Fig. 1). Although SecYEG (Baba *et al.* 1990; Koch and Muller 2000) and YidC (Samuelson *et al.* 2000; Chen *et al.* 2002; Dalbey, Koch and Kuhn 2017) can act as independent insertion sites for membrane proteins, they also cooperate during membrane protein insertion (Scotti *et al.* 2000; Beck *et al.* 2001; Houben *et al.* 2004; Zhu *et al.* 2012; Sachelaru *et al.* 2013; Komar *et al.* 2016; Sachelaru *et al.* 2017; Petriman *et al.* 2018). Here, YidC facilitates the release of transmembrane domains (TMs) from the SecYEG channel (Beck *et al.* 2001), supports their folding (Zhu, Kaback and Dalbey 2013; Serdiuk *et al.* 2016) and controls the correct topology of membrane proteins (Nagamori, Smirnova and Kaback 2004).

The SecYEG channel is also engaged in the post-translational transport of secretory proteins, which are targeted by the ATPase SecA after their release from the ribosome (Muller *et al.* 2001; de Keyzer, van der Does and Driessen 2003; Alami *et al.* 2007; Fig. 1). SecA exhibits a dual function during transport of secretory proteins; it serves as a targeting factor, which requires for some substrates the cooperation with the cytoplasmic chaperone SecB (Castanie-Cornet, Bruel and Genevaux 2013), and it provides the energy for translocation by repetitive ATP hydrolysis cycles (Knyazev *et al.* 2018). While the SRP-dependent targeting to the Sec translocon is universally conserved and essential in almost all cells (Dalbey, Koch and Kuhn 2017), the SecA-dependent targeting is present in bacteria and chloroplasts only and absent in eukaryotes and archaea (Pohlschroder *et al.* 2005).

Structure of the bacterial SRP and its receptor FtsY

The bacterial SRP is a ribonucleoprotein complex that contains a single and highly conserved GTPase subunit that is homologous to the eukaryotic SRP54 subunit and thus named Ffh (Fifty-four-homologue; Miller, Bernstein and Walter 1994). Ffh is bound to either 4.5S RNA in gram-negative bacteria or to 6S RNA in gram-positive bacteria (Rosenblad *et al.* 2003; Fig. 2). Accessory proteins interacting with the bacterial SRP have been described for *Bacillus subtilis* and *Streptococcus mutans*. In *B. subtilis*, the histone-like protein HBsu was suggested to be part of SRP (Nakamura *et al.* 1999), but *in vitro* analyses did not reveal significant binding of HBsu to SRP (Beckert *et al.* 2015). YlxM was identified as component of the *S. mutans* SRP and seems to regulate GTP hydrolysis (Williams *et al.* 2014). However, the exact functions of these accessory proteins have to be further explored.

The bacterial SRP receptor (SR) FtsY is also composed of a single GTPase subunit. Although most bacterial SR lack a TM, they are tightly bound to the cytoplasmic membrane by virtue of lipid- and SecY-binding sites (Mircheva *et al.* 2009). Besides Ffh and FtsY, some bacterial species contain a third SRP-like GTPase, termed FlhF (Bange *et al.* 2007; Bange *et al.* 2011), which appears to be dispensable for protein transport (Zanen *et al.* 2004), but rather controls the number and position of flagella (Schuhmacher, Thormann and Bange 2015).

Both FtsY and Ffh consist of three domains each and their respective N- and G-domains show striking similarities in terms of architecture and amino acid sequence (Fig. 2). The N-domain forms a bundle of four helices that is followed by the Ras-like GTPase G-domain (Freymann *et al.* 1997; Montoya *et al.* 1997). Different to many other GTPases, Ffh and FtsY are stable in the absence of nucleotides and show only small conformational changes upon nucleotide binding (Freymann *et al.* 1997; Montoya *et al.* 1997; Freymann *et al.* 1999; Gawronski-Salerno and Freymann 2007). Their intrinsically low GTPase activity is stimulated upon Ffh–FtsY complex formation via their NG domains. This forms a composite GTP-hydrolysis site that promotes reciprocal GTP hydrolysis at the end of the targeting reaction (Kusters *et al.* 1995; Egea *et al.* 2004; Focia *et al.* 2004; Bange and Sinning 2013; Fig. 2).

The non-homologous third domains of Ffh and FtsY execute particular functions during the targeting reaction. The C-terminal methionine-rich M-domain of Ffh is flexibly connected to the NG domain by a 30 amino acid-long linker and serves as binding site for signal sequences and contacts the SRP RNA (Spanggord *et al.* 2005; Halic *et al.* 2006; Janda *et al.* 2010; Hainzl *et al.* 2011). Five amphipathic α-helices (αM1 to αM5) at the C-terminus of the M-domain form a hydrophobic signal sequence binding groove, which can be closed by a finger-loop located between αM1 and αM2 (Hainzl *et al.* 2011) and the flexible αM5 (Jomaa *et al.* 2016).

The third domain in FtsY is the N-terminal A-domain, containing a large number of acidic residues. While the M-domain in Ffh is absolutely essential for SRP function, the A-domain is present primarily in FtsY homologues of gram-negative bacteria and absent in gram-positive bacteria and atypical bacteria like *Mycoplasma*, *Mycobacterium* or *Spirochaetes* (Kakeshita *et al.* 2000; Dong, Jiang and Li 2009). Even in gram-negative bacteria there is limited sequence and length conservation of the A-domain and deleting the A-domain does not prevent targeting *in vivo* and *in vitro* (Eitan and Bibi 2004; Weiche *et al.* 2008). Although not essential, the 198 amino acid long A-domain in *Escherichia coli* executes important functions. It contains an amphipathic lipid-binding helix at the very N-terminus (Weiche *et al.* 2008; Braig *et al.* 2009) and binding sites for the SecYEG translocon (Angelini, Deitermann and Koch 2005; Kuhn *et al.* 2011; Kuhn *et al.* 2015). The C-terminus of the A-domain also facilitates the folding of a second amphipathic lipid binding helix at the interface of the A- and N-domains (Stjepanovic *et al.* 2011). Thus, the A-domain serves to stabilize the essential membrane contact of FtsY (de Leeuw *et al.* 2000; Mircheva *et al.* 2009; Erez *et al.* 2010; Lam *et al.* 2010). Even in its absence, the essential lipid-binding helix at the A-N interface (Parlitz *et al.* 2007; Braig *et al.* 2011; Stjepanovic *et al.* 2011) and additional SecY-binding sites within the NG domains (Kuhn *et al.* 2015) are sufficient for maintaining efficient co-translational targeting. Recent data demonstrate that the A-domain also prevents futile SRP–FtsY interactions in the absence of an available SecYEG translocon (Draycheva *et al.* 2016). This is in line with a recent Cryo-EM structure, in which a domain presumably reflecting the A-domain was localized close to the SecYEG translocon (Jomaa *et al.* 2017). Why in particular gram-negative bacteria require the A-domain remains an open question.

The third component of the SRP system is the SRP RNA. The RNA is essential for SRP function, but differs remarkably in size and structure. Initially considered to merely serve as a scaffold for Ffh, it is now evident that the RNA is a catalytic component that regulates GTP hydrolysis of the SRP–FtsY complex (Peluso *et al.* 2001; Ariosa *et al.* 2013). The 4.5S SRP RNA of *E. coli* is composed of 115 nucleotides and forms a hairpin-like structure (Jagath *et al.* 2001; Gu *et al.* 2005) with six internal loops and the conserved GGAA tetra-loop at the closed end (Fig. 2). The loops A and B are located close to the tetra-loop and bind to a helix-turn-helix motif within the M-domain of SRP. The SRP RNA of many gram-positive bacteria is larger (270 nucleotides in *B. subtilis*) and contains in contrast to gram-negative bacteria an Alu-domain (exceptions are e.g. *Mycobacterium tuberculosis* or *Corynebacterium glutamicum*; Regalia, Rosenblad and

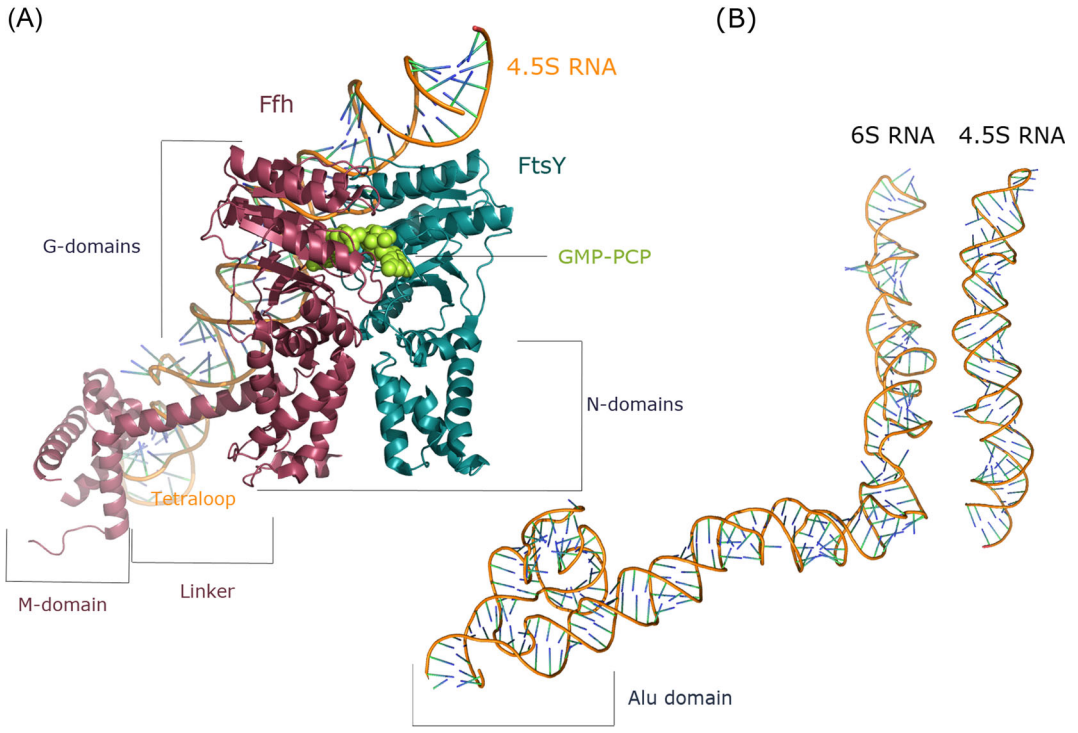


Figure 2. Structure of the bacterial SRP components. (**A**) Crystal structure of *E.coli* SRP in complex with its receptor FtsY (Ataide *et al.* 2011, pdb: 2XXA). Ffh, the GTPase protein subunit of SRP is displayed in red, in complex with the NG domains of its receptor FtsY, depicted in blue. The non-hydrolysable GTP analogue GMPPCP (green) is also indicated. A 30 amino acid long linker connects the NG-core of Ffh to its C-terminal methionine rich M-domain that provides the binding sites for the SAS and the SRP RNA, shown in green. The tetra-loop of 4.5S RNA regulates GTP hydrolysis of the SRP–FtsY complex. The last C terminal 21 amino acid residues of Ffh are not depicted. The 198 amino acid long acidic A-domain of FtsY, present primarily in gram-negative bacteria, is disordered and has not been crystallized so far. (**B**) Comparison of *Deinococcus radiourans* 4.5S RNA (Ataide *et al.* 2011, pdb: 2XXA) and *B. subtilis* 6S RNA (Beckert *et al.* 2015, pdb: 4UE4). The 4.5S RNA of gram-negative bacteria is about half the size of the 6S RNA, present in most gram-positive bacteria. The Alu-domain of the 6S RNA, which is involved in translation regulation, is indicated.

Samuelsson 2002). This domain decelerates translation, likely via competition with elongation factor binding to the ribosome (Beckert *et al.* 2015), thus providing a time window for efficient membrane targeting of RNCs by SRP (Koch, Moser and Muller 2003). Although shorter and lacking the Alu-domain, the 4.5S RNA can complement the depletion of the 6S RNA in *B. subtilis* and restore viability (Nakamura *et al.* 1992).

In contrast to the Alu-domain of the eukaryotic SRP, the bacterial Alu-domain is protein-free with large RNA-extensions (Kempf, Wild and Sinning 2014), and the translational arrest activity of the *B. subtilis* Alu-domain largely depends on RNA-RNA interactions (Beckert *et al.* 2015). Different to the SRP of eukaryotes and gram-positive bacteria, a translational arrest activity by SRP in gram-negative bacteria has not been described, even though SRP and its sub-domains reduce membrane protein expression when over-expressed (Yosef, Bochkareva and Bibi 2010). An alternative model for adjusting translational speed to co-translational targeting is codon-usage. Indeed, local slow-down of translation by non-optimal codons and translational pause sites promote RNC recognition by SRP in bacteria and eukaryotes (Chartier, Gaudreault and Najmanovich 2012; Fluman *et al.* 2014; Pechmann, Chartron and Frydman 2014).

Ribosome binding and substrate selection by SRP

Co-translational targeting is initiated by SRP contacting the ribosome for subsequent decoding of the information retained within the emerging nascent protein. On the ribosomal surface, SRP establishes three contacts next to the ribosomal tunnel exit (Halic *et al.* 2006; Schaffitzel *et al.* 2006; Jomaa *et al.* 2016), which seem to be conserved across species: the NG domain binds in close vicinity to the ribosomal proteins uL29 and uL23 (Pool *et al.* 2002; Gu *et al.* 2003; Jomaa *et al.* 2016). uL23 is also contacted by the M-domain (Halic *et al.* 2006; Schaffitzel *et al.* 2006; Jomaa *et al.* 2016) that furthermore extensively interacts with the 23S rRNA of the bacterial ribosome. The 4.5S RNA of SRP contacts bL32, a ribosomal protein that is only found in bacteria (Jomaa *et al.* 2016; Fig. 3).

Membrane proteins contain a unique identification tag at the N-terminus, called the signal anchor sequence (SAS), which distinguishes them from secretory proteins and cytosolic proteins (Driessen and Nouwen 2008). The SAS is not a defined amino acid sequence, but rather a consecutive stretch of hydrophobic and bulky aromatic residues. These residues are flanked by basic residues at the N-terminal side and polar residues on the C-terminal side (Pugsley 1990; von Heijne 1994; Hegde and Bernstein 2006). As such, these SAS appear to be not much different from signal sequences of secretory proteins. Indeed, it is largely the increased hydrophobicity and the absence of helix-breaking amino acids (Lee and Bernstein 2001; Adams *et al.* 2002; Beha *et al.* 2003; Zhang *et al.* 2010; Petersen *et al.* 2011) that distinguishes SAS from signal sequences. In addition and reflecting its particular name, SAS lack a signal peptidase cleavage site and usually serve as first TM for anchoring the protein in the membrane. In contrast, signal sequences of secretory proteins are usually cleaved after translocation by signal peptidase-1 (Hegde and Bernstein 2006). Finally, recent data

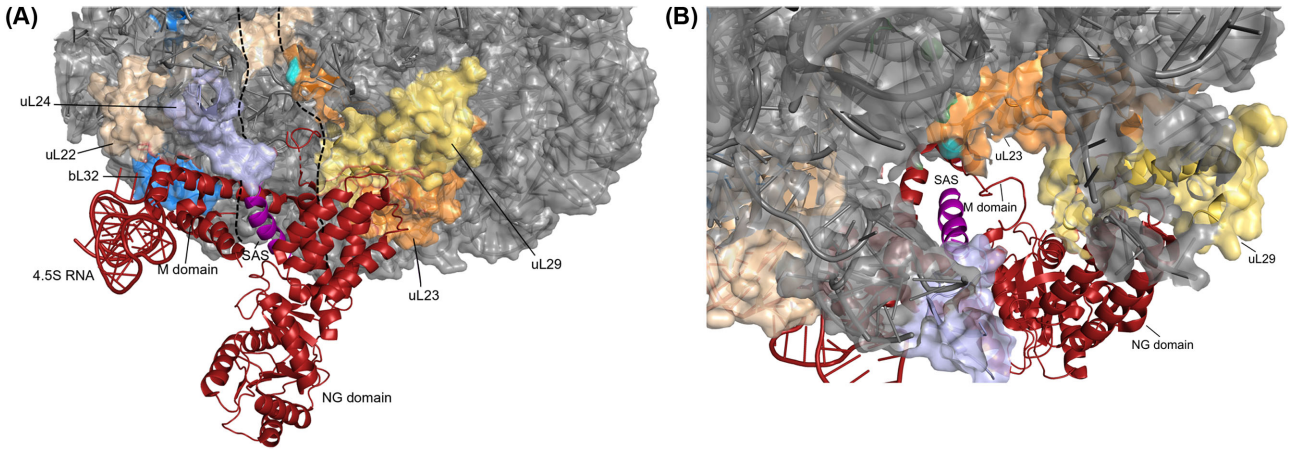


Figure 3. Atomic model of the SRP–RNC interaction. Cryo-electron microscopy structure of the SRP–RNC complex (adapted from Jomaa *et al.* 2016; pdb: 5GAF). (**A**) The ribosomal surface is shown in gray and the ribosomal peptide tunnel is marked with dashed lines. The ribosomal proteins uL22 (wheat), uL23 (orange), uL24 (light violet) and uL29 (yellow) are located at the lower part of the ribosomal tunnel and surround the ribosomal tunnel exit. The NG and M-domain, as well as the 4.5S RNA of SRP, are displayed in red. The ribosomal protein bL32 (blue) is contacted by the 4.5S RNA, whereas uL23 and uL29 are interacting with both the M- and NG domains of SRP. The signal anchor sequence (SAS) is shown in purple. The C-terminus of the M-domain extends into the ribosomal tunnel getting close to the intra-tunnel hairpin loop of uL23 (position 71 at the loop tip is marked in green). Note that helix αM5 at the very C-terminus of the Ffh M-domain, which was shown to interact with position 71 of uL23 by cross-linking (Denks *et al.* 2017), is not depicted in the structure. (**B**) Top view of the ribosomal tunnel. The SAS as well as the loop of the C-terminal M-domain of Ffh (lacking the αM5 helix) are located inside of the ribosomal tunnel.

demonstrate that information within the mature part of secretory proteins also contributes to their efficient targeting (Chatzi *et al.* 2017). Whether SRP-dependent targeting is also influenced by sequence information down-stream of the SAS is currently unknown.

SRP contacts to both the ribosome and to the SAS are prerequisites for co-translational targeting, but the timing and coordination of these contacts were subjects of controversial discussions. The initial proposal based on work with eukaryotic SRP suggested that SRP identifies its substrates after the SAS is completely exposed to the outside of the ribosomal exit tunnel (Walter, Ibrahimi and Blobel 1981). Recent re-investigations using single-molecule fluorescence energy transfer to monitor SRP-binding to actively translating bacterial ribosomes and ribosome profiling data supported these earlier findings (Noriega *et al.* 2014; Noriega *et al.* 2014; Schibich *et al.* 2016). However, various other studies demonstrated a much earlier interaction of the eukaryotic and bacterial SRP with non-translating ribosomes (Bornemann *et al.* 2008) and short RNCs in which the nascent protein was completely shielded within the ribosomal tunnel (Houben *et al.* 2005; Berndt *et al.* 2009; Voorhees and Hegde 2015). Even membrane targeting was shown to occur without a fully exposed SAS (Bornemann *et al.* 2008; Holtkamp *et al.* 2012). Recent kinetic analyses describe the SRP–RNC interaction as a multistep process and find early SRP recruitment before emerging of a SAS using both stalled RNCs and actively translating ribosomes (Bornemann *et al.* 2008; Holtkamp *et al.* 2012; Mercier *et al.* 2017). A first moderate-affinity intermediate SRP–RNC complex is detected early and independently of the presence or exposure of a SAS. This intermediate is rapidly followed by a rearrangement to a more stable complex in which SRP awaits the emerging SAS (Mercier *et al.* 2017). Once the SAS is completely exposed, i.e. when the nascent chain reaches a length of approx. 45 amino acids, the affinity for SRP is further increased (Denks *et al.* 2017), resulting in a kinetically stable complex that can initiate the subsequent delivery to FtsY. This increase in stability of SRP in complex with ribosomes bearing a nascent chain, compared to SRP binding to non-translating ribosomes, is mainly mediated by a decrease in the dissociation rate (1 s^{-1} versus 10 s^{-1}) of SRP from the ribosome (Bornemann *et al.* 2008; Holtkamp *et al.* 2012). The early interaction between SRP and the ribosome is therefore characterized by a high dissociation rate that could reflect an early rapid scanning mode of SRP for possible substrates. This might explain how the small amount of SRP molecules can efficiently scan the large excess of ribosomes in the bacterial cell. When contacting translating ribosomes, the dissociation rate is lowered as the nascent chain further emerges, switching to a stand-by complex that probably primes SRP for binding the SAS. As soon as a SAS is recognized, the stability is further increased (dissociation rate: 0.08 s^{-1}) and targeting is initiated (Bornemann *et al.* 2008; Holtkamp *et al.* 2012).

In vivo and *in vitro* crosslinking data confirm the concept of an early, SAS-independent interaction of SRP with RNCs. In these studies it was shown that the C-terminus of Ffh (helix αM5) inserts into the ribosomal tunnel of non-translating ribosomes to contact an intra-tunnel hairpin-loop of the ribosomal protein uL23 (Denks *et al.* 2017; Fig. 4). Once the nascent chain reaches a length of approx. 25 amino acids, SRP retracts from the intra-tunnel loop into the proximal part of the tunnel and the intra-tunnel loop now contacts the nascent chain. Importantly, this step was independent of a SAS. The retraction of the C-terminus probably orients SRP in such a way that the emerging SAS now makes stable contact with the C-terminal M-domain (Denks *et al.* 2017). This activates SRP for the subsequent binding to FtsY (Bornemann *et al.* 2008; Buskiewicz *et al.* 2009). The insertion of the C-terminus of Ffh into the ribosomal tunnel was also visible in a recent high-resolution Cryo-electron microscopy study (Jomaa *et al.* 2016), providing further evidence for this model. In the absence of a correct SAS, SRP dissociates and scans the next ribosome. A recent study on SRP–RNC interaction in yeast also supports early ribosome binding of SRP, but further suggests that non-coding regions within the mRNA also influence SRP recruitment (Geiger *et al.* 2016). The discrepancy between the SAS-dependent and -independent SRP-binding models is probably explained by the low stability of early SRP–ribosome interactions. In particular, the cross-linking approach as non-equilibrium method favors the detection of labile intermediates by covalent stabilization.

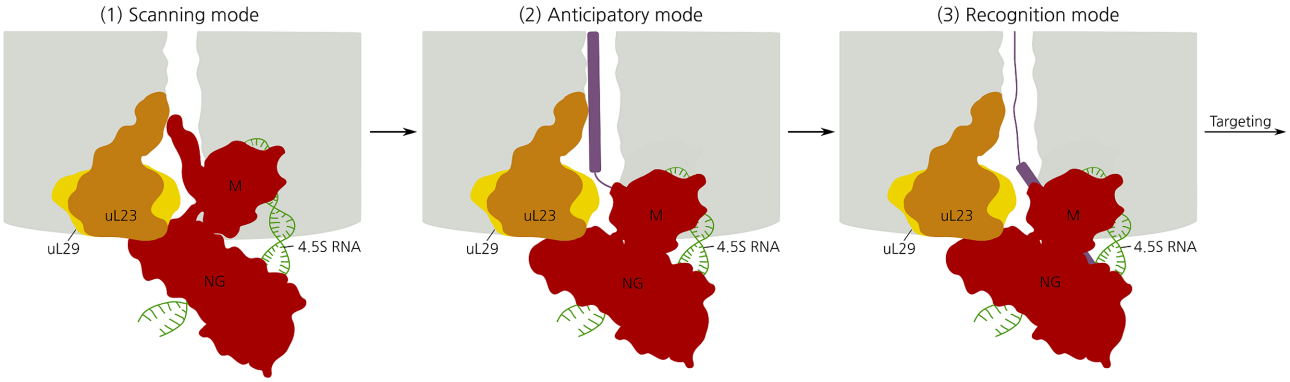


Figure 4. Schematic view of the different interaction states between SRP and ribosomes during different stages of substrate recognition (modified from Denks *et al.* 2017). When contacting vacant ribosomes (light gray), the flexible C-terminal part of the M-domain of SRP (red) is inserted into the ribosomal tunnel and contacts the loop-tip of the ribosomal protein uL23 (orange). This reflects an early scanning mode of SRP. That contact is replaced by the emerging nascent chain (purple) and SRP retracts into the proximal part of the tunnel anticipating an emerging SAS. Once the SAS is completely or almost completely exposed, stable binding of SRP to the SAS enables SRP to subsequently target the RNC to the SecYEG-bound SR FtsY.

Expectedly, there are deviations from the canonical system described above (Table 1). SRP sometimes fails to recognize the first TM but rather binds a downstream TM or it binds to membrane protein substrates multiple times (Schibich *et al.* 2016). As skipped TMs generally have a lower hydrophobicity, the interaction of SRP with these TMs might be too weak to be detected. Multiple binding events on the other hand could suggest re-targeting of RNCs that have lost contact to the SecYEG translocon or YidC during insertion (Kuroiwa *et al.* 1996). Some membrane proteins have cleavable signal sequences, like the ammonium transporter AtmB (Khademi *et al.* 2004), and their targeting and insertion mode is unexplored. In other membrane proteins, like KdpD, the first TM is preceded by a long cytosolic domain, which contains amphipathic stretches that are recognized by SRP (Maier *et al.* 2008), demonstrating that SRP recognition is not limited to TMs. This is also supported by the observation that SRP can bind to particularly hydrophobic signal sequences of secretory proteins and even to amphipathic helices of cytosolic proteins (Huber *et al.* 2005; Zhou, Ueda and Muller 2014; Schibich *et al.* 2016). Among the cytosolic proteins bound by SRP are the partially membrane associated proteins DnaK (Schibich *et al.* 2016) and σ^{32} (Lim *et al.* 2013). *Escherichia coli* contains a few tail-anchored proteins (Borgese and Righi 2010), i.e. proteins that are membrane-anchored by a C-terminal TM that is only accessible after translation termination. Their targeting and insertion pathway is still largely unexplored, but SRP is involved in their transport as well (Pross *et al.* 2016; Peschke *et al.* 2017). Finally, bacteria contain an increasing number of small membrane proteins (<50 amino acids; Fontaine, Fuchs and Storz 2011; Storz, Wolf and Ramamurthi 2014). These are suggested to depend on either YidC or SecYEG for insertion (Fontaine, Fuchs and Storz 2011), but whether they require SRP/FtsY is unknown.

Targeting of nascent membrane proteins for insertion

After substrate recognition, SRP delivers its cargo to the cytoplasmic membrane via the interaction with its receptor FtsY. The timing of this SRP–RNC–FtsY interaction was also initially controversially discussed, owing to the fact that FtsY, in contrast to the eukaryotic SR, lacks a TM that would tether it permanently to the cytoplasmic membrane in bacteria. Cell fractionation studies had shown that FtsY is found at the membrane and in the soluble fraction (Luirink *et al.* 1994), raising the possibility that FtsY would first associate with the SRP–RNC in the cytosol and only then target the membrane (Saraogi, Akopian and Shan 2014). In support of this assumption, the FtsY association with SRP or SRP–RNCs in solution has been shown in multiple studies and was crucial for the biochemical and structural characterization of distinct conformational changes during the targeting reaction (Jagath, Rodnina and Wintermeyer 2000; Ataide *et al.* 2011; Estrozi *et al.* 2011; von Loeffelholz *et al.* 2013; von Loeffelholz *et al.* 2015). Still, the lipid- and SecY-binding sites in FtsY tether it almost exclusively to the membrane *in vivo* (Mircheva *et al.* 2009) and the association of FtsY with lipids (Lam *et al.* 2010; Braig *et al.* 2011; Stjepanovic *et al.* 2011) and the SecYEG translocon (Angelini, Deitermann and Koch 2005; Akopian *et al.* 2013; Kuhn *et al.* 2015; Draycheva *et al.* 2016) is a prerequisite for high-affinity SRP binding. The preference of FtsY for anionic phospholipids (de Leeuw *et al.* 2000; Braig *et al.* 2009; Erez *et al.* 2010; Lam *et al.* 2010), which are enriched in close vicinity to the SecYEG translocon (Gold *et al.* 2010; Prabudiansyah *et al.* 2015), likely enhances targeting efficiency by increasing the local concentration of FtsY close to SecYEG.

FtsY binds to the cytosolic loops C4 and C5 of SecY and thus occupies the ribosome-binding site of the SecYEG translocon (Kuhn *et al.* 2011). Binding occurs with high affinity (K_D = 0.18 μM) and is lipid-dependent as described above, but nucleotide-independent (Kuhn *et al.* 2015). Considering approx. 10 000 FtsY copies and approx. 500 SecYEG copies per *E. coli* cell (Kudva *et al.* 2013), only a fraction of FtsY can be in direct contact with SecY. The number of FtsY molecules in complex with SecYEG is further reduced because SecA and ribosomes bind to the same cytosolic loops of SecY (Kuhn *et al.* 2011). Thus, contacts between SecYEG and FtsY are likely highly dynamic and the FtsY molecules not bound to SecYEG are bound to either phospholipids (Weiche *et al.* 2008; Braig *et al.* 2009) or YidC (Welte *et al.* 2012; Petriman *et al.* 2018). The contact of FtsY to SecYEG induces the movement of the A-domain away from the NG domain resulting in the exposure of the SRP-binding site on FtsY (Draycheva *et al.* 2016). This observation is consistent with earlier observations that only the SecYEG-bound FtsY is competent for efficient SRP recruitment (Mircheva *et al.* 2009). This domain separation is one of several check-points during the SRP-targeting cycle that ensures effective substrate delivery to SecYEG (Bornemann *et al.* 2008; Holtkamp *et al.* 2012; Saraogi, Akopian and Shan 2014). However, it is important to emphasize that the A-domain is not conserved in all bacterial species and therefore the SRP-binding site of FtsY in these species is not

Table 1. Diversity of targeting and membrane insertion strategies for membrane proteins (MPs) in bacteria.

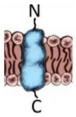
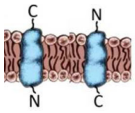
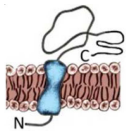
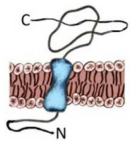
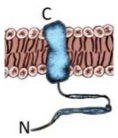
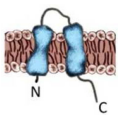
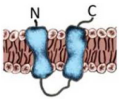
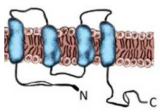
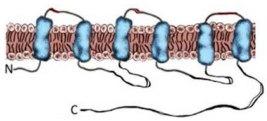
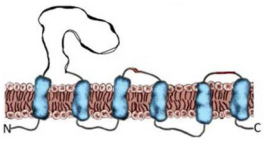
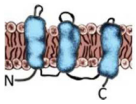
Topology	Type and examples	Targeting		Insertion/Translocation			References
		SRP	SecA	Sec YEG	YidC	SecA	
	**Phage proteins**						
	Pf3coat	+	–	–	+	–	Chen *et al.* (2002)
	S-MPs[a]						
	YoaJ	?	?	+	+	?	
	YkgR	?	?	+	–	?	Fontaine, Fuchs and Storz (2011)
	YoaK	?	?	–	+	?	
	YohP	?	?	–	–	?	
	**Single-spanning MPs**						
	FtsQ	+	–	+	*	+	van der Laan, Nouwen and Driessen (2004)
	RodZ	–	+	+	?	+	Rawat *et al.* (2015); Wang, Yang and Shan (2017)
	**C-tail anchored MPs**						
	SciP, Flk, DjlC	+	–	–	+	–	Pross *et al.* (2016); Peschke *et al.* (2017)
	**Double-spanning MPs**						
	MscL	+	–	?[b]	+	–	Facey *et al.* (2007) Komar *et al.* (2016)
	F_0c	?[c]	–	?[d]	+	–	Yi *et al.* (2004); van Bloois et al (2004); Komar et al (2016)
	**Multi-spanning MPs**						
	KdpD	+	–	–	–	–	Maier *et al.* (2008); Facey *et al.* (2003)
	MtlA	+	–	+[e]	+[e]	–	Koch *et al.* (1999); Welte *et al.* (2012)

Table 1. *continued*

Topology	Type and examples	Targeting		Insertion/Translocation			References
		SRP	SecA	Sec YEG	YidC	SecA	
	YidC	+	–	+	–	+	Koch *et al.* (2002); Welte *et al.* (2012)
	TatC	+	–	+[e]	+[e]	–	Welte *et al.* (2012); Zhu *et al.* (2012)

(Steinberg *et al.*, Table 1)

Shown are the demonstrated or predicted topologies of different types of membrane proteins and their experimentally verified targeting and insertion mechanism. (+) required; (∗) involved, e.g. YidC involvement during folding of SecYEG-inserted membrane proteins; (–) not required; (?) unknown or conflicting data.

[a]S-MPs refer to small membrane proteins of less than 50 amino acids; their insertion was studied with SPA-tagged variants.

[b]In a reconstituted system, a SecYEG-mediated insertion of MscL was shown (Komar *et al.* 2016).

[c,d]Both SRP-dependent (van Bloois *et al.* 2004) and in-dependent targeting (Yi *et al.* 2004) of F_0c was shown; slightly higher insertion efficiency was observed with a SecYEG–YidC–SecDF complex (Komar *et al.* 2016).

[e]SRP can target MtlA and TatC to either SecYEG or YidC for insertion (Welte *et al.* 2012).

shielded. Whether these bacteria use other means to prevent SRP–FtsY interaction in the absence of an available SecYEG translocon is unknown.

The activation of FtsY by lipid-embedded SecYEG and the activation of SRP by binding to the SAS guide into the next step of the targeting reaction: the formation of a quaternary SecYEG–FtsY–SRP–RNC complex (Saraogi, Akopian and Shan 2014; Kuhn *et al.* 2015; Jomaa *et al.* 2017). This involves a series of conformational rearrangements, which finally lead to the reciprocal GTPase activation. However, it is important to note that the early conformational FtsY–SRP rearrangements were determined only in solution, i.e. in the absence of membranes and the SecYEG translocon. First, the SRP–RNC and the SR form an unstable intermediate (early intermediate) that is based on electrostatic interactions between the respective N-domains of FtsY and Ffh (Estrozi *et al.* 2011; Zhang *et al.* 2011) and on contacts between the tetra-loop of 4.5S RNA and FtsY (Jagath *et al.* 2001; Zhang, Kung and Shan 2008). In this early interaction state incorrect substrates can still be recognized and rejected from the SRP pathway. In this case the SRP–RNC–FtsY complex dissociates (von Loeffelholz *et al.* 2013). In presence of a correct substrate, the complex is stabilized to a closed state mediated by conformational changes of the respective NG domains (Egea *et al.* 2004; Shan, Stroud and Walter 2004). As a consequence of this FtsY–SRP interaction, FtsY is partially displaced from SecY, leading to the exposure of the ribosome binding site on SecY. Likewise, SRP movements on the RNCs expose the translocon-binding site on the ribosome (Pool *et al.* 2002; Halic *et al.* 2006), which favors the stable contact between the RNC and the SecYEG translocon and the subsequent insertion of the nascent membrane protein into the lipid bilayer. The concomitant GTP-hydrolysis by the SRP–FtsY complex finally leads to its disassembly (Kusters *et al.* 1995; Akopian *et al.* 2013; Bange and Sinning 2013; Voigts-Hoffmann *et al.* 2013), releasing SRP into the cytosol, whereas FtsY remains bound to SecYEG or lipids (Kuhn *et al.* 2015; Mercier *et al.* 2017).

SRP and FtsY deliver substrates not only to the SecYEG translocon but also to the YidC insertase (Dalbey, Koch and Kuhn 2017). This was shown for proteins like MscL or F_0C that are suggested to strictly require YidC for insertion (van Bloois *et al.* 2004; Facey *et al.* 2007) and for proteins like MtlA or TatC that can be inserted *in vitro* by either SecYEG or YidC (Welte *et al.* 2012; Table 1). Thus, SRP does not necessarily discriminate between SecYEG and YidC substrates, which would be anyway difficult to imagine as SRP binds to its substrates before substantial information about the substrate is available. RNCs bind to the cytosolic loop C2 and the C-terminus of YidC (Kohler *et al.* 2009; Kedrov *et al.* 2013; Kedrov *et al.* 2016), but the affinity of YidC for non-translating ribosomes is rather low compared to SecYEG (Welte *et al.* 2012; Kedrov *et al.* 2013). Only YidC variants with C-terminal extensions have a high intrinsic affinity for non-translating ribosomes. These YidC variants are found in some marine bacteria (Seitl *et al.* 2014) and in some gram-positive bacteria like *Streptococcus* (Hasona *et al.* 2005; Dong *et al.* 2008; Funes *et al.* 2009), and are termed YidC2. Strikingly, *S. mutans* is one of the few organisms that survives the inactivation of the SRP pathway (Hasona *et al.* 2005), likely because the C-terminus of YidC2 provides enough specificity for ribosome binding to maintain cell survival even in the absence of a targeting system.

YidC also binds FtsY and SRP (Welte *et al.* 2012), but SecYEG appears to be the preferred binding partner of FtsY (Petriman *et al.* 2018). This is in line with the observation that protein insertion into YidC-proteoliposomes does not strictly require FtsY (Welte *et al.* 2012), while FtsY is absolutely essential for insertion into SecYEG-proteoliposomes (Braig *et al.* 2011). Further studies are required for determining whether SRP-dependent targeting to YidC mimics the targeting to SecYEG or shows differences. Variations in the SRP cycle and FtsY requirements during targeting to SecYEG or YidC could explain data indicating alternative ways of ribosome-targeting to the bacterial membrane (Bibi 2011). In this scenario, FtsY is co-translationally targeted to the membrane (Bercovich-Kinori and Bibi 2015), whereby the pool of membrane-bound ribosomes is increased (Herskovits *et al.* 2001; Herskovits *et al.* 2002; Bahari *et al.* 2007). These ribosomes could translate mRNAs encoding membrane proteins that were targeted to the membrane independently of translation (Nevo-Dinur *et al.* 2011), but the subsequent insertion would still require SRP (Yosef, Bochkareva and Bibi 2010).

SecA, another ribosome-interacting targeting factor?

SecA-dependent targeting of secretory proteins is generally considered to occur post-translationally (Lee and Beckwith 1986; Mori and Ito 2001; Chatzi *et al.* 2013), but this view is challenged by several observations. The interaction of SecA with RNCs of secretory proteins was first observed in cell-free *in vitro* studies (Eisner *et al.* 2003; Karamyshev and Johnson 2005) and a dual role of SecA in the targeting/insertion of short RNCs and during the translocation of periplasmic loops of membrane proteins was also noticed (Neumann-Haefelin *et al.* 2000; van der Laan, Nouwen and Driessen 2004; Deitermann, Sprie and Koch 2005; Fig. 5). Later, SecA was shown to bind to the ribosome in close vicinity of the ribosomal tunnel (Huber *et al.* 2011; Singh *et al.* 2014) and to long RNCs of the secretory maltose-binding protein *in vivo* (Huber *et al.* 2017). The observation that the SecA interaction requires extended nascent chains (>110 amino acids) is interesting, as this is also the RNC length that allows maximal interaction of the chaperone trigger factor (Oh *et al.* 2011), which is generally considered to be the first contact of a nascent secretory protein (Kramer *et al.* 2002; Ito 2005; Calloni *et al.* 2012).

The early interaction of SecA with secretory proteins is yet another example that living cells usually do not follow black-and-white classifications. This is even more evident when looking at recent data which demonstrate that SecA is also involved in the co-translational targeting and insertion of the single-spanning membrane protein RodZ (Rawat *et al.* 2015; Wang, Yang and Shan 2017), confirming previous data on a potential role of SecA in the insertion of single-spanning membrane proteins (Deitermann, Sprie and Koch 2005). This suggests an alternative co-translational route for membrane protein targeting in bacteria that involves SecA and is independent of SRP; however, to which extent this pathway is used and which determinants route membrane proteins into this SRP-independent targeting pathway needs to be further explored. Considering that SecA binding to SecYEG follows the same principle as FtsY binding to SecYEG (Lill, Dowhan and Wickner 1990; Kuhn *et al.* 2011) and that both proteins bind SecY with similar affinities (Douville *et al.* 1995; Kuhn *et al.* 2015), SecA-dependent targeting of nascent membrane proteins might act as back-up system when the low-abundant SRP system is saturated.

Biotechnological and medical relevance of co-translational targeting

Heterologous expression of membrane proteins is still a major bottle-neck for their structural and biochemical characterization. Many attempts to facilitate membrane protein expression in *E. coli* actually rely on attenuating expression for preventing membrane stress, e.g. by lowering the amounts of T7 RNA polymerase or by reducing temperature (Wagner *et al.* 2008; Baumgarten *et al.* 2017) or on *E. coli* strain optimization (Schlegel *et al.* 2010; Kuipers *et al.* 2017). Improving heterologous membrane protein expression by modulating their targeting and insertion might be a promising alternative. Increasing the cellular YidC levels improved the expression of two archaeal rhodopsins in *E. coli*, but increasing the SRP concentration reduced their yield (Nannenga and Baneyx 2011). Reduced membrane protein expression upon SRP-overproduction was also observed for endogenous membrane proteins (Yosef, Bochkareva and Bibi 2010) and was suggested to prevent the overflow of the limited SecYEG channels (Nannenga and Baneyx 2012). This could reflect a translational arrest activity of the *E. coli* SRP, but the underlying mechanism is unknown. Over-expression of SecYEG is usually also not well tolerated, but enhanced production of vitamin K epoxide reductase was observed when the substrate-binding groove of YidC was modified (Hatahet *et al.* 2015). Thus, improving YidC-dependent membrane protein folding and computationally guided sequence optimization are promising ways for improving membrane protein expression (Norholm *et al.* 2012; Norholm *et al.* 2013; Marshall *et al.* 2016; Niesen *et al.* 2017).

Exploring the protein targeting and transport machinery as targets for novel antibacterials has gained momentum during the last years (Rao *et al.* 2014; Van Puyenbroeck and Vermeire 2018). Three major targets have so far been identified: the motor protein SecA (Chaudhary *et al.* 2015; De Waelheyns *et al.* 2015), signal peptides (De Waelheyns *et al.* 2015) and the SecYEG translocon (Junne *et al.* 2015). Even though specific inhibitors of SRP, FtsY and YidC could not be isolated, down-regulation of YidC sensitizes bacterial cells against antimicrobial compounds (Patil *et al.* 2013), and inhibitors of the RNC transfer from the SRP machinery to the Sec translocon in eukaryotes have been described (Cross *et al.* 2009). The therapeutic potentials of these compounds have to be further explored, but they definitely offer exciting new avenues for treating bacterial infections.

CONCLUSION AND OUTLOOK

Research on bacterial protein transport has seen tremendous progress during the last years. The structures of all of the key components have been determined and, in combination with detailed biochemical analyses, this now permits a deep insight into the molecular processes that ensure the specific substrate delivery to the bacterial cytoplasmic membrane. And yet, many questions are still unresolved and new questions arise. The co-translational targeting by SRP and FtsY is almost exclusively studied in *Escherichia coli* by using a limited number of model substrates. Its contribution to the transport of non-canonical substrates, like small membrane proteins (Storz, Wolf and Ramamurthi 2014), C-tail-anchored proteins (Pross *et al.* 2016; Peschke *et al.* 2017) and proteins that seem to insert independently of SecYEG or YidC (Facey and Kuhn 2003) needs to be further explored. Ribosome profiling studies have determined the global SRP interactome in *E. coli* (Schibich *et al.* 2016) that provides the framework for further validation, but also needs to be extended to other species. Studies on the SRP-dependent targeting to YidC (Welte *et al.* 2012) are still in its infancy and it remains to be seen whether it indeed follows the pathway explored for targeting to SecYEG. The influence of translational speed and codon usage on SRP recognition (Pechmann, Chartron and Frydman 2014) is another topic that has been barely touched. The ribosomal tunnel exit is a rather crowded place and multiple targeting factors, chaperones and processing factors bind to it; how their access to the nascent protein is coordinated is still subject of ongoing research (Ito *et al.* 2011; Sandikci *et al.* 2013; Bornemann, Holtkamp and Wintermeyer 2014; Ranjan *et al.* 2017). The SRP pathway is considered essential to most bacteria, but then how do bacteria like *Leptospira sp.* survive in the absence of an SRP system (Fouts *et al.* 2016)? Alternative targeting strategies for membrane proteins have recently emerged and the importance of the SecA–ribosome interaction and the prevalence of SecA-dependent membrane protein targeting is another unresolved issue (Huber *et al.* 2017; Wang, Yang and Shan 2017). Likewise, the translation-independent membrane targeting of mRNAs encoding membrane proteins and the transfer of their products to SecYEG or YidC for insertion are open questions (Bibi 2011). Finally, the spatial organization of the targeting and insertion

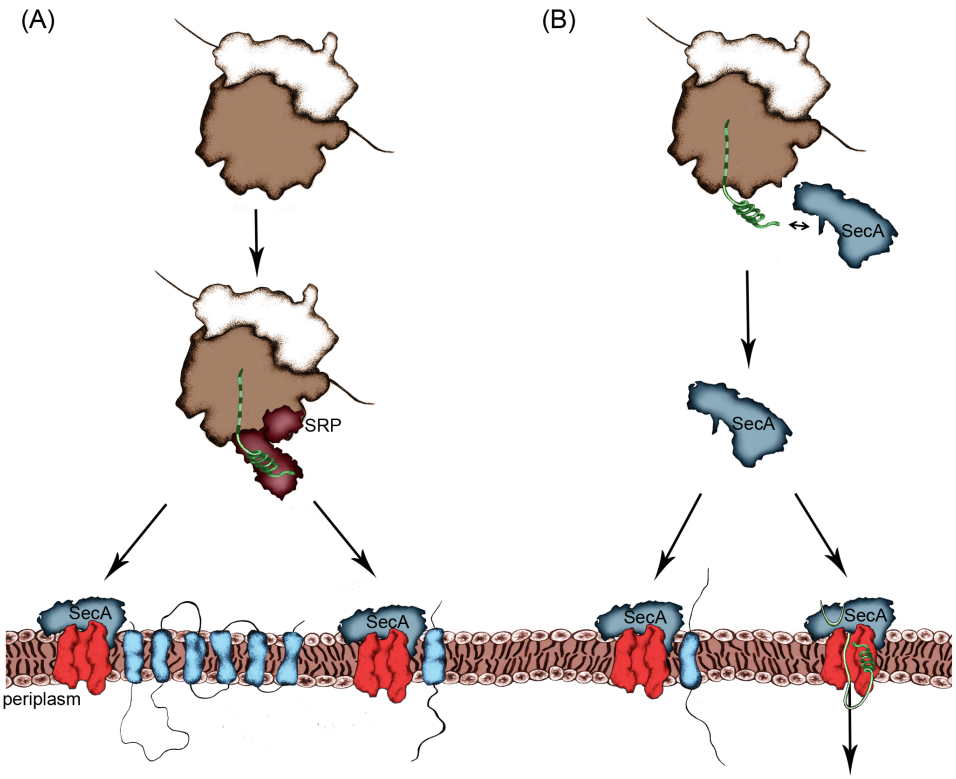


Figure 5. The contribution of SecA to membrane protein insertion in *E. coli*. (**A**) Membrane proteins harboring extended periplasmic domains require SecA for the translocation of these loops. This has been shown for multi-spanning membrane proteins like YidC (left panel) and for single-spanning membrane proteins like FtsQ (right panel). These proteins are targeted co-translationally by SRP to the SecYEG translocon (red). Whether SecA (blue) translocates the periplasmic loops after synthesis is terminated or during synthesis is not known. (**B**) SecA can also recognize its substrates co-translationally. This has been shown for the single-spanning membrane protein RodZ (left panel), which is targeted and inserted independently of the SRP pathway. Whether canonical SecA substrates like periplasmic proteins (right panel) can also be recognized and targeted by SecA co-translationally is currently unknown.

machinery in bacterial cells is only beginning to emerge (Campo *et al*. 2004; Govindarajan and Amster-Choder 2017). Thus, despite all the progress, bacterial protein transport remains a fascinating topic for research that holds the premise for more surprises.

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