# Cellular localization of bacterial Sec translocase

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## Abstract

The Sec pathway in bacteria facilitates secretory and membrane protein translocation across or into the cytoplasmic membrane. In the post-translational pathway, the protein chaperone SecB addresses secretory proteins to the pore forming SecYEG complex. The ATPase SecA provides energy that is needed for protein excretion through this pore. In the co-translational pathway, membrane proteins are recognized by the Signal Recognition Particle (SRP) which also directs to the SecYEG complex. Opening of a lateral gate in the pore facilitates insertion of the membrane protein in the membrane. The membrane associated proteins YidC and SecDFyajC support protein translocation.

Extensive research has been performed on cellular localization of the primary components of the Sec system, SecA and SecYEG. Localization of the Sec components are thought to be related with sites of cell wall synthesis, as well as distribution of anionic lipids over the membrane. In rod-shaped gram-positive bacteria, as well as in gram negative bacteria, these factors induce a helical distribution of the Sec proteins over the membrane. In coccus-shaped gram-positive bacteria, it is thought that the Sec machinery is located at the septum, or in a specific site near the septum: the ExPortal.

However, research techniques varied in different studies. In many researches, Sec proteins were overexpressed. Also different microscope techniques were used to visualize the proteins. Recent research on localization of the Sec translocation machinery gave aberrant results, which could be due to use of new techniques (genomic integration and TIRF or PALM microscopy). A better representation of the living cell can be generated with these techniques, which makes them promising for further research on localization the Sec pathway.

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## 1. Introduction

About one third of bacterial proteins has to be inserted into or excreted through the (inner) cell membrane in order to fulfill their function. This process of transporting target proteins can be carried out by multiple mechanisms, of which the Sec pathway is most common. The basis of this secretion and membrane insertion machinery is the membrane spanning SecYEG protein complex, which forms a selective pore in the (cytoplasmic) bacterial membrane [1].

A close look into bacterial life shows that almost all processes are very well orchestrated, in order to function properly. There are many examples of membrane proteins that are precisely localized into the pole, septum or in another fashion over the cytoplasmic membrane [2]. In this literature research, an attempt is made to sort out the hypotheses about the arrangement of the Sec translocon over the cytoplasmic membrane of different bacteria species.

## 2. Sec targeting pathway

Proteins are synthesized at the ribosome, which translates the mRNA of secretory proteins into a protein. Secretory and integral membrane proteins have a tag in their DNA sequence. This so called signal sequence can be seen as an address label. The difference between the tag of secretory and membrane proteins defines their translocation pathway and destination. The sequence tag is located on the N-terminal side of the protein and consists of three regions: the N-region that has positive amino acids, the H-region that has hydrophobic amino acids, and the C-region that contains a cleavage site [1]. After or during translation, the protein can be recognized by either the protein chaperone, e.g. SecB, which targets secretory proteins via the post-translational pathway, or the Signal Recognition Particle which targets membrane proteins via the co-translational pathway. Both pathways lead to the pore forming complex SecYEG, which is located in the cytoplasmic membrane. The ATPase protein SecA is the energy source for this process. By hydrolyzing ATP it pushes the protein through the gate. After translocation through the membrane, the protein folds into its final three-dimensional structure (figure 1) [3].

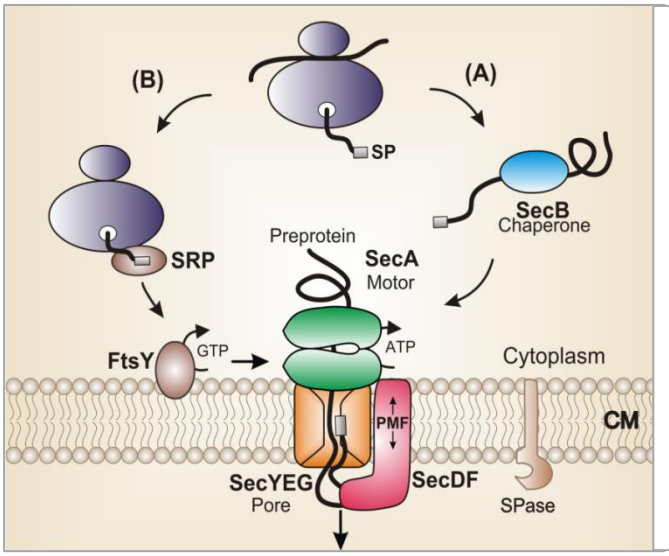


**Figure 1:** The Sec pathway. (A) Post-translational targeting: preproteins synthesized at the ribosome (purple) are captured in an unfolded state by a chaperone protein (blue), and targeted to SecA (green) that is bound to the SecYEG channel (orange) at the cytoplasmic membrane (CM). SecA pushes the preprotein through the SecYEG channel in an ATP-dependent manner. In addition, SecDFyajC (red) uses the PMF to pull preproteins into the periplasm. After translocation, the signal peptide (SP) is removed by signal peptidase (SPase) and the mature protein is released. (B) In co-translational targeting, preproteins are targeted to the translocation site as a ribosome-nascent chain complex (RNC) by SRP and FtsY (brown) where protein synthesis will commence involving SecA and ATP.

## 2.1. Post-translational pathway

Secretory proteins are transported across the inner membrane via the *post-translational pathway,* i.e. they are fully synthesized prior to transport. In this targeting pathway, the proteins are in contact with the molecular chaperones which maintain them in a translocation-competent state in the cytosol. In proteobacteria, this chaperone function is carried out by SecB. SecB is a dimer of dimers with a total molecular weight of 69 kDa. The SecB chaperone binds secretory proteins with low specifity but high affinity and can handle proteins up to 150 amino acids in length. The proteins wrap around the chaperone in a 1:1 ratio. This prevents initial folding into a three-dimensional structure. The binding of protein increases the affinity of the negative beta-sheets of SecB to the C-termini of secA, which has a positive charge due to basic amino acids and a bound Zinc-atom [4]. After a ternary SecA-SecB-secretory protein complex is formed, the complex docks to secYEG, the pore in the membrane. SecA hydrolizes ATP in order to give the energy that is required to push the unfolded protein through the SecYEG complex [5], [6].

## 2.2. Co-translatonal pathway

Apart from the post-translational pathway that transports secretory proteins, there is a pathway for membrane proteins: *the co-translational pathway*. In eukaryotes, a ribosome translating a protein with a membrane signal sequence is recognized by the Signal Recognition particle (SRP). SRP is a protein that ensures membrane insertion. In *Escherichia coli* this protein is not present, but its function is taken over by the SRP homolog, a cytosolic signal sequence binding subunit Ffh. Ffh (fifty four homologue) interacts with ribosomes translating membrane preproteins. It also has a hydrophobic pocket containing a conserved region of methionines, which prevents initial folding of the protein. The complex is recognized by the receptor FtsY, which is embedded in the cytosolic membrane. FtsY couples the ribosome-Ffh complex to the SecYEG complex which can insert the preprotein into the membrane [7].
The signal sequence of membrane preproteins, also termed stop-transfer sequence, has a big effect on the SecYEG complex: it prevents translocation and supports opening of a side-exit: the lateral gate. This process is independent of the ATPase activity of SecA. The membrane insertion rate of the translocated protein is determined by hydrophobicity of the protein itself (a larger hydrophobic segment will insert more easily into the membrane) [8].

# 3. Structure and function of Sec component

The Sec translocase pathway involves a large group of proteins, of which the structure and mechanisms will be explained in this paragraph. The most interesting proteins for this research are SecA and the SecYEG complex, since they are involved in both translocation routes and are essential for survival.

## 3.1 SecA

SecA is a highly conserved bacterial protein and functions as an ATP-driven molecular motor to facilitate protein translocation across the SecYEG protein-conducting pore (figure 2). The ATPase SecA forms a dimer of 102 kDa monomers in active translocation. Most of SecA dimers are organized as an anti-parallel, for example in *Escherichia coli* and *Bacillus subtilis* [6][9] but SecA from *Thermus Hermophilus* revealed a parallel conformation [10]. Each monomer has four structural domains: two nucleotide binding sites are facing the outside of the structure, where they can easily bind ATP. [6] Two ATPase sites that contain a DEAD-motor, also found in DNA helicases, facilitate and control ATP hydrolysis. A C-terminal domain is important in binding a preprotein carrying SecB, as well as binding

to the membrane via anionic phospholipids [1]. In protein translocation, a region of a SecA monomer binds to SecYEG. Afterwards, another SecA monomer binds to the complex, which makes the complex functionally active. Binding and hydrolysis of ATP triggers a conformational changes in the SecA structure, resulting in insertion of the secretory/membrane protein in steps of 20 to 25 aminoacids [3].

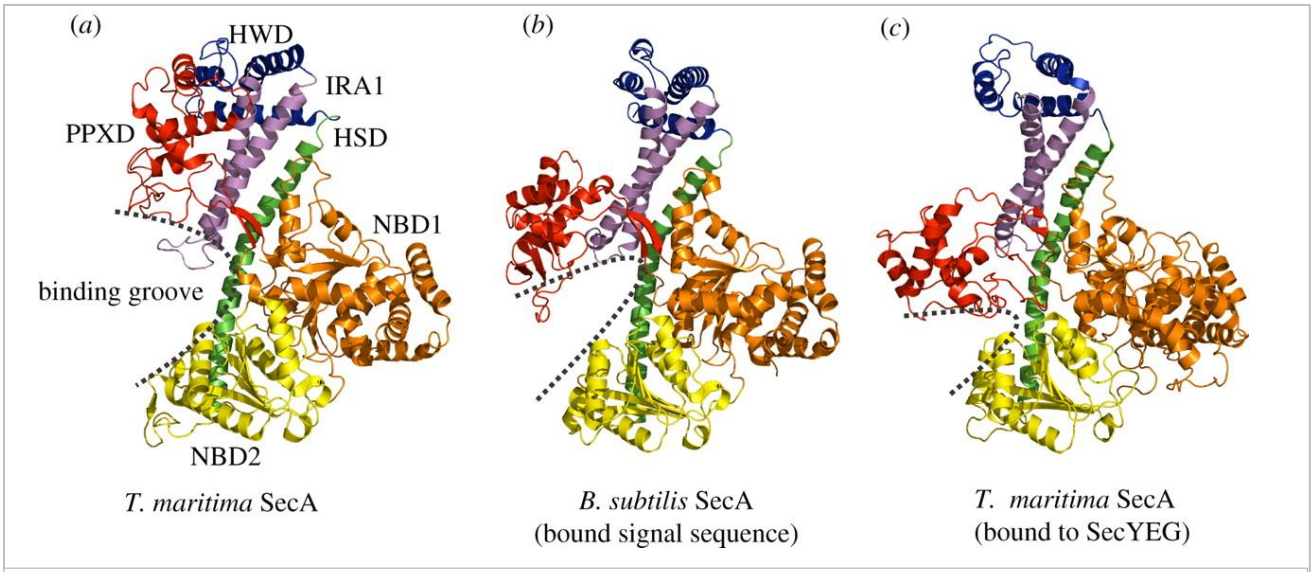


**Figure 2.** Structure of SecA, the translocation ATPase. (A) in Thermotoga maritima (PDB accession code 4YSO). Nucleotide Binding Domains in orange and yellow. The ATPase site in Purple (IRA1, intramolecular region of ATP hydrolysis). Proposed preprotein binding groove in dashed lines. (B) SecA in B. subtilis (PDB accession code 1M6N). (C) T. maritima SecA when bound to SecYEG (SecYEG not shown)
*source: The bacterial Sec-translocase, structure and mechanism (2012), J. A. Lycklama Nijeholt and A.J.M Driessen* [3]

## 3.2 SecYEG

SecYEG is a protein complex built of three integral membrane proteins: SecY, SecE and SecG (figure 3). The complex is homologous to Sec61αγβ in eukaryotes and SecYEβ in archaea[1]. SecY is a 48 kDa protein that forms the actual pore in the membrane. Ten membrane spanning helices form a hourglass shaped tunnel, with in the middle a circle of isoleucines forming the pore ring. An alpha helix at the periplasmatic side of the protein can fold back into the structure, forming a plug capable of closing the pore [11]. The 14 kDa protein SecE has a close interaction with SecY and is like a clamp around the pore. Its main goal is to prevent SecY from degradation by the membrane protease FtsZ. Both SecY and SecE are essential for survival [12]. SecG is a small 12 kDa protein, which is not essential and of which the exact function remains unknown. However, it is found in all homologues of the bacterial Sec system [11].

There has been some discussion about oligomerization of the SecYEG complex. Most studies suggest a dynamic equilibrium of SecYEG monomers, dimers and tetramers. Translocation seems to promote oligomerization of the active YEG complexes into tetramers, with two dimers having the lateral gates facing each other. Also the ideal ratio between SecYEG and SecA, obtained by quantitative western blotting, is 2:1. This confirms the idea that SecYEG forms tetramers, interacting with dimers of SecA. Translocation seems to promote oligomerization of the SecYEG complex [13][14].

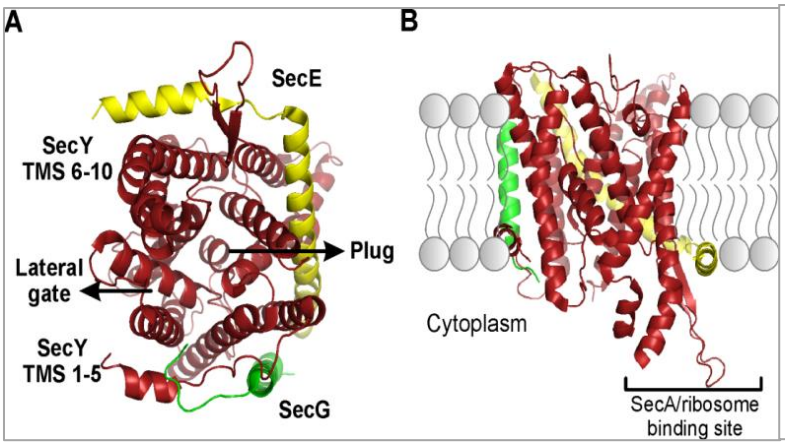


**Figure 3**: Structure of SecYEG, the protein conducting channel. (A) Structure of SecYEβ from Methanoccoccus jannaschii (PDB accession code: 1RH5), viewed from the cytoplasm. SecY is coloured in red, SecE in yellow, and SecG in green. The lateral gate and the plug are indicated with the arrow line. (B) SecYEβ viewed from the side, in position in the lipid bilayer.

### 3.3 YidC and SecDFyajC

The translocation process is not only driven by ATP hydrolysis but has supplemental support of the Proton Motive Force (PMF). The heterodimeric membrane protein SecDF is associated with SecYEG. One domain of SecDF points into the periplasm and can bind to the emerging preprotein. SecDF has its head domain pointed in the periplasm near the exit of the secY pore, thereby being able to interact with the assembling secretory preprotein. By transporting protons over the membrane (PMF), it generates energy to make a rotational movement, thereby promoting protein translocation and preventing backward motion. The small protein YajC is thought to have a scaffolding role in this process by connecting SecDF to SecYEG [3].

The membrane protein YidC supports insertion and proper folding of certain, mostly large, membrane proteins like F1F0 ATPase [15]. Sec DF also plays a role membrane insertion by YidC, acting like a scaffold to couple YidC to SecYEG [3].

## 4. Cellular localization of the Sec machinery

Prokaryotes seem to be very simple organisms: organelles are not present and the genomic information is not separated from the rest of the cell. It looks like a bacterium is just a bag of DNA and proteins. However, a close look reveals a very precise and structured organization. Membrane proteins form no exception to this: localization of membrane proteins in bacteria is far from random. Various research has been applied on localization of the membrane bound parts of the Sec machinery. In this literature research, the main focus will be on the primary components of the Sec-system, SecA and SecYEG.

### 4.1 Different localization strategies

A process in which cellular localization is essential, is cell division. The FtsZ protein is known for initiating cell division by making a contractile ring in mid cell, which eventually splits the cell in two halves. But how does it define the middle of the cell? In E.coli, the Min system is responsible for this. The FtsZ inhibitors MinC and MinD cluster at one pole, travel to the other pole, cluster again, etc. A ring of MinE forms the rim of the MinCD clusters. In order to pass MinE to maintain the oscillational movement from pole to pole, MinCD needs to disassemble from the membrane. This results in a

lower concentration of MinCD in mid-cell membrane, which means less inhibition for FtsZ, resulting in a cell division site right at the middle of the cell [16].

Apart from mid cell localization, there are proteins that are localized in the poles. For example, chemoreceptors need to be at one pole for effective chemotaxis. Also formation of stalks, pili, flagella or other mechanisms used for motility typically appear at one pole. IscA in the pathogen *Shigella flexneri* recruits actin from host cells for motility. IcsA a distinct preference for the 'old' pole (which was already a pole before cell division), but mechanisms behind this are still unclear [2].

There are also examples of proteins localizing in a helical array. The cell shape determining protein MbI in B. subtilis forms actin like cables in a helical organization. Absence of this protein makes the cell grow in a less efficient, pole to pole or division site based mode of growth. MbI governs cell wall growth in a direct manner: it actively directs the building of new peptidoglycan, somehow recruiting new monomers to be secreted by the cell in order to insert it in the membrane. This results in a helical growing pattern of peptidoglycan [17].

## 4.2 Factors determining cellular localization

Cellular localization can be dependent of many factors. Cell cycle and related events such as peptidoglycan recruitment seem to be a key player in this process, but also protein-protein interactions such as receptor/ligand interactions, cleavage or inhibition/activation play their part. For the Sec machinery, the peptidoglycan cell wall seems to be important for protein translocation.
It is known that the peptidoglycan layer is built op from the lateral side of the protein [2]. This means that the poles are the more static regions of the cell, in contrast to the midcell.
Also the composition of lipids in the cell membrane may play a role in cellular localization of membrane proteins. There are different kinds of lipids in the membrane, and there is great heterogeneity between bacterial species in the relative proportion and the localization of these different kinds of lipids [18]. It has been showed that the anionic phosphatidylglycerol forms spiral like structures in the cell [19]. Other research pointed out that the anionic phospholipid Cardiolipin, is localized at the poles of the cells. Anionic lipids are known to be associated with ATP-ase activity and protein translocation, and therefore they are thought to play a role in cellular localization of Sec [18].

## 4.3 Localization of the Sec translocase in rod shaped gram positive bacteria

Gram positive bacteria only have one membrane, unlike gram-negative bacteria that have two membranes with a stable periplasmic environment in between. This gives several challenges to secretion proteins. At first, the extracellular environment is unpredictable and quite different from the homeostatic cytoplasm. Also accessory proteins are absent outside the cell [20]. Furthermore, the cell wall has permeability for proteins up to 25 to 50 kDa, larger proteins will have a big challenge passing it [21].
In *B. subtilis*, it was not clear for a long time what the distribution of SecA and SecYEG across the membrane was. Research of Campo et al. (2004) in *B. subtilis* suggested that SecA is arranged in a helical pattern. In this study SecA and SecY were fused to GFP in a plasmid under control of a xylose inducible promotor. Based on 3D reconstructions of deconvolution microscopy, it could be concluded that three to ten Sec clusters are arranged into two spiral like structures. SecY and SecA localize in a similar pattern, however SecY shows more clustering than SecA.
Immunogold labeling and immunofluorescence microscopy on SecY showed similar results. A study

with deletion strains of MbI showed that this helical distribution does not depend on the presence of the cell shape determining MbI: the helical distribution of SecY remained present, but to a less extent. The authors suggest that the relation between MbI and the Sec translocase is the other way around: MbI might be dependent of the Sec machinery [22].
Rafelski and Theriot (2006) suggested that there is a correlation between protein secretion and cell wall synthesis [23]. Sec translocons might be fixed in space, while the cell wall grows around them. This would give an advantage in protein secretion: at cell wall synthesis sites, the cell wall is the thinnest so it will be easiest to pass. Furthermore it creates more periplasmic space for the protein to fold. This hypothesis is strengthened by the finding that SecA localization is dependent of active protein transcription and translation [22]. Also, since new peptidoglycan monomers are inserted from the inside, the synthesis of the cell wall itself could help pushing secretory proteins through the wall into the external medium [21].
It has also been showed that the localization of anionic phospholipids may be important in Sec dependent protein translocation. Since they support SecA increasing ATPase activity, it is thought that anionic phospholipids play a role in localization of the Sec machinery. Phosphatidylglycerol forms lipid spirals, which is consistent with the theory that the Sec machinery has a helical organization. This hypothesis is enhanced by deletion studies: strains depleted of this type of phospholipids don't show a helical distribution of SecA anymore [18] [22] .

A very recent research (Dajkovic et al, June 2016) gave new insight into the localization of SecA and SecY in *B. subtilis* . This was done by using a new microscopy technique called TIRF-Microscopy on SecA-GFP and SecY-GFP fusions, integrated in the genomic DNA of *B. subtilis*. Fluorescence correlation spectroscopy based analysis on the microscope movies provided insights in the dynamics of the proteins. Surprisingly, it was found that SecA as well as SecY is dynamically distributed over the cell membrane. Furthermore, the location of the translocon is not fixed over time, resulting in a random assembling and disassembling of oligomers over time. No helical patterns were detected [24]. It seems strange that two similar researches give such different results. The different outcome is most likely due to a different technique of expressing the GFP-Sec protein fusion. While Campo et al. uses a plasmid based system, while Dajkovic et al. integrates the fusion into the genomic DNA.
Rubio et al (2005) also used genomic integration of GFP-protein fusions for research into other components of the Sec pathway in B. subtilis. The YidC homologues SpoIII and YgjG were found to be evenly distributed over the membrane, as well as SecDF, which is suggested to associate with SecYEG [25].

## 4.4 Localization of the Sec translocase in coccus shaped gram positive bacteria

Although cocci have a similar cell wall structure as rod-shaped gram positive bacteria, mechanisms be fairly distinct. Rod shaped bacteria have two modes of cell wall formation: the first mode is responsible for forming the cell division (orchestrated by FtsZ), the second one (orchestrated by MbI) is responsible for elongation of the cell and shows helical localization. However cocci only have mechanisms that insert cell wall proteins in the septum area, which implies that a helical organization of the Sec machinery seems unlikely, because organization of the Sec machinery seemed quite dependent on cell wall synthesis in rod shaped gram positive bacteria [21].
Research *in Streptococcus pyogenes* revealed protein secretion takes place in one specific area: the Exportal, which seems to be a specialized microdomain or organelle for protein secretion. The study was not focused on Sec but on secreted proteins. It showed with immunolabeling experiments as

well as a protease assays that the frequently occurring native secreted protein SpeB, its maturation protein HtrA and the non native PhoZ were exclusively secreted from and localized in this organelle[20] [21]. Every cell has one ExPortal that is localized in close proximity to a forming septum. This specific localization of the Sec machinery may again be related to cell wall synthetis. FtsZ, mentioned before, is involved in cell division, but is also in charge for localizing new peptidoglycan in the cell wall. The ExPortal is localized exactly where FtsZ is localized, which could mean that the cocci, like rod shaped gram positive bacteria, localize to the place where protein translocation through the thick cell wall is easiest. So again, the localization of Sec machinery seems to use the formation process of the cell wall as a driving force to push newly synthesized proteins though the thick peptidoglycan[26]. Also this specific domain is rich in anionic lipids[18].

Research in *Streptococcus pneumoniae*, however, suggests that in this bacterial species SecA and SecY are localized in the whole septum ring. The study integrated FLAG tagged SecA and SecY into the chromosome of S. pneumoniae and followed expression in different growth stages using IFM (Immunofluorescence Microscopy). It shows that the Sec proteins primarily localize in the septum of predivisional cells and in one pole (former septum) in postdivisional cells. After cell division, the Sec proteins redistribute towards the new septum. Since cell wall synthesis takes place in the whole septum ring, these findings seem to follow to the theory of Sec machinery localization near sites of cell wall synthesis [27].

### 4.5 Localization of the Sec translocase in gram negative bacteria

Apart from gram-positive bacteria, gram negative bacteria have a very thin cell wall. Also these bacteria have two membranes, which means that proteins are secreted in the relatively stable periplasmic space. Based on the knowledge from localization of the Sec translocon in gram-positive bacteria, these factors imply that a random distribution over the membrane would be plausible. Indeed, an in vivo fluorescence microscopy study in *E. coli* from Branden et al. (2003) using C-terminally GFP labeled of SecY and SecE show that the translocon is uniformly distributed over the cytoplasmic membrane. Proteins were slightly overexpressed in this study [28]. However, research from Shiomi et al. in 2006 employing fluorescence microscopy and 3D reconstitution using a SecE-GFP construct showed a helical distribution of the Sec translocon. In this study the protein was not overexpressed.. This finding was confirmed by Immunofluorescence microscopy on SecG, and by co-localization study with the fluorescently labeled SecYEG substrate (CFP-MalE and YFP-Tar). Just as in *B. Subtilis*, this helical distribution was different from the coiling of the MbI like MreB, but it could be dependent of another, not yet discovered, cytoskeletal protein. Also could the phospholipid composition or cell even cell wall synthesis direct the Sec translocon in this distribution [29]. In *E.coli* the phospholipid cardiolipin might play a role in distribution of the SecYEG complex.. Deletion studies showed that helical organization of the complex disappeared after deletion of cardiolipin. This is strange however, because cardiolipin is localized at the poles of the cell [18].

## 5. Conclusion

The Sec translocase has been discovered and studied for quite some years now. Structures have been described and this gave perfect insight in the mechanisms of protein translocation across the bacterial membrane. Two routes were discovered: the post-translational route for secretion proteins via SecB and the co-translational route for membrane proteins via the SRP. Both routes use the SecA as a molecular motor for protein translocation through the SecYEG translocon, which structure is conserved in all three domains of life.

Spacial distribution of the sec machinery over the cytoplasmic membrane, however, seems not to be so straight forward and varies between different bacterial species. In vitro and in vivo studies show that localization of the Sec machinery is most likely dependent of two factors. At first: the cell wall. This seems to play a big part in gram positive bacteria, because the cell wall of these species is very thick and difficult to pass for secreted proteins. It seems likely that the Sec machinery is localized at places were this barrier is past most easily: sites of cell wall synthesis. The cell wall is at it thinnest on these sites, and insertion of new peptidoglycan from the inside promotes secretion of proteins. In rod shaped bacteria such as *B. subtilis*, cell wall synthesis takes place in an helical arrangement, and indeed, *in vivo* studies show a helical arrangement of the Sec machinery. In coccus shaped bacteria it is thought that the Sec translocon is localized either in the septum, or in a special domain called ExPortal near the septum. Also this is according to the cell wall synthesis theory. In gram negative bacteria, the cell wall is much thinner, which suggest that it doesn't play a big role. The opposite seems to apply, because it is highly plausible that also gram negative bacteria (*E.coli*) have their Sec translocons in a helical distribution.

This brings us to the next candidate for determining localization of the Sec machinery: phospholipid composition. Anionic phospholipids promote ATPase activity of SecA and protein translocation, so it will be likely that the Sec translocon will be localized in close proximity of these lipids. Indeed, the *B. subtilis* Sec translocon is dependent on these phospholipids, according to deletion studies. The ExPortal in coccus shaped bacteria is rich in anionic phospholipids, and in *E.coli* the helical organization of SecA seems somehow dependent on cardiolipin.

## 6. Discussion and future perspectives

The two theories mentioned before seem to explain the localization of Sec quite well and seem to enhance each other. Case closed, one could think. However, the techniques used in the various studies are not interchangeable, and this may be the reason that there is no consensus about the cellular localization pattern of the Sec machinery. Not every technique will give a good representation of the actual situation in the cell.

In many studies on cellular localization, proteins are made visible with a fluorescence (GFP) tag. This fusion product can be expressed in a plasmid, coupled to a non-native inducible promotor. Expression levels can be regulated from high (overexpression) to low expression (close to native), but will always show localization of proteins additional to the proteins expressed by the genomic DNA. It would be better to visualize the native protein, for example by replacing the gene at the native locus for a GFP fusion product. This technique, called homologous recombination, visualizes proteins under

a native promotor in native concentrations in the cell [30]. In section 4.3 is discussed how these two techniques can result in different outcomes. For experiments on localization, genomic integration techniques seem to be the best approach for getting reliable results that represent the situation in a living cell. This would undermine the theory of a helical distribution of Sec proteins in *B. subtilis*.

Furthermore, visualization techniques are becoming more advanced. TIRF (total internal reflection fluorescence) microscopy makes it possible to track single molecules with resolution in the nanometer range. At the moment, it is the "method of choice for single molecule tracking on extended substrates" [31]. PALM (photo activated localization microscopy) is a technique based on TIRF microscopy. It is capable of generating images of fluorophores in a resolution of less than 10 nm. The technique is based on activation and bleaching of fluorophores. The image of the fluorophores can be analyzed by fitting Gaussian functions to it, after which exact location and oligomerizaton state can be derived. Another adventage of this microscope technique is that it is possible to analyze living cells. While in IFM (immunofluorescence) and other techniques the cells need to be fixed into place, in PALM experiments flow cells can be used to study living cells [27], [32].

The mentioned techniques make it possible to look into the cellular dynamics of life. Further research could focus on these techniques in order to give a clear and final conclusion about the localization of membrane proteins, such as the Sec translocon.

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