Benefits and challenges of the secretory pathways of *Bacillus subtilis* to biotechnology

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Abstract

Heterologous protein production using microbial hosts is a burgeoning field of biotechnology which promises to revolutionize many sectors of industry. This rapid innovation has been largely fueled by fundamental knowledge combined with improved genetic engineering tools. One of the most promising workhorses for heterologous protein expression is the Gram-positive bacterium *Bacillus subtilis*, favored for its capacity to secrete large amounts of protein. In this review the molecular mechanisms behind protein secretion in *B. subtilis* are discussed. First, the two main protein secretion pathways of *B. subtilis*, Sec and Tat, are discussed. Subsequently, chaperone activity and extracellular proteases, and their effect on protein secretion are dealt with. The last part of this review looks at the ways these components have been altered by molecular biologists in recent years in order to optimize *B. subtilis* as a protein expression host. Together this will provide an overview of the accomplishments made with *B. subtilis* as well as the challenges remaining.

Introduction

The industrial production of proteins has become very important for the pharmaceutical and nutrition sectors of industry. Expression of heterologous proteins with the help of microbial hosts has driven down costs and increased production capacity of many industrially useful proteins. Still, many proteins have not been produced in sufficient quantities yet and increasing protein yields is a prospect that has drawn a wide interest from experts in the field of biotechnology. In the past decades, advancing microbiological knowledge has led to many successful attempts to increase heterologous protein expression, but getting yields of heterologous protein as high as those obtainable for homologous protein has remained largely elusive. Extracellular production of proteins reduces the purification steps needed to get the protein of interest from the cells of the expression host. Because of this secretion of heterologous proteins has largely been preferred over cytoplasmic production.

The Gram-positive bacterium *Bacillus subtilis* has been widely used for heterologous protein production due to its high capacity for protein secretion, ranging from 20 to 25 g/L (van Dijl & Hecker, 2013). For a long time most heterologous protein expression was performed with *Escherichia coli* because of its well established genetic engineering tools and status as bacterial model organism. However, many proteins could not be expressed in *E. coli* (Terpe, 2006). Moreover, many genetic sequences of heterologous proteins must be altered to match the codon bias of *E. coli* while *B. subtilis* does not have such a pronounced codon bias (Luan & Yang, 2019). The biggest advantage of *B. subtilis* over *E. coli* is that it does not produce lipopolysaccharides, which trigger immune responses in humans, thereby making it suitable for the production of proteins destined for pharmaceutical or nutritional products (van Tilburg *et al*., 2019).

Other protein expression hosts include eukaryotic cells, methylotrophic bacteria and other members of the *Bacillus* genus. Among eukaryotic hosts, filamentous fungi are most widely used and offer better folding and quality control of heterologous proteins. In addition they can perform certain post-translational modifications, which are often essential for the functioning of eukaryotic proteins (Nevalainen *et al*., 2005). Methylotrophic bacteria are cheap to grow and are capable of producing significant quantities of protein, but have not been used to produce many proteins to date (Terpe, 2006). *Bacillus megaterium* has low protease activity, high plasmid stability and can grow on many substrates. Although *B. subtilis* is quite proteolytic, strains have been engineered to have low protease and high plasmid stability as well (Terpe, 2006).

Because *B. subtilis* is a well-established laboratory organism many genetic engineering tools are available for improving heterologous protein production and secretion. The publication of the full sequence of the *B. subtilis* genome proved to be an enormous boon to fundamental research on gene expression and protein production and subsequent engineering efforts to improve these systems (Kunst *et al*., 1997). More recently, the emergence of CRISPR/Cas9 as a cheap and efficient gene-editing tool has made metabolic engineering of *B. subtilis* much easier (Hong *et al*., 2018). Low yields of proteins in *B. subtilis* have been attributed to weak promoters, poor ribosome binding sequences and low plasmid copy numbers (Song *et al*., 2016). The strong constitutive P43 and SPO bacteriophage promoters are the most widely used promoters for increasing protein expression (Cui *et al*., 2018). Many more strong promoters that are useful for heterologous protein expression have been found and engineered by screening large libraries and placing promoters in tandem (Song *et al*., 2016; Cui *et al*., 2018). Optimizing

transcription and translation of heterologous protein can be used as an alternative to optimizing secretion pathways to improve heterologous protein expression but the former are beyond the scope of this review. Another approach for creating expression host strains has been to knock out genes related to sporulation, autolysis and proteolysis (Cui *et al*., 2018). Gene knock out technology has been used for decades to create increasingly specialized strains for heterologous protein expression, starting with a strain deficient in six extracellular proteases and having recently lead to optimized strains for the production of specific proteins (Wu *et al*., 1991; Zhao *et al*., 2018). Recently developed systems have optimized *B. subtilis* for pharmaceutical or nutritional purposes in an entirely different way. One study made *B. subtilis* suitable for food-grade protein production by developing a stable plasmid that does not rely on antibiotic resistance for screening. Using a genome-incorporated toxin and an antitoxin carried by a plasmid that also contains a gene of interest, the plasmid remained present in the population for over 100 division cycles (Yang *et al*., 2016). Another study employed biofilms produced by *B. subtilis* to present antigens that can be used for medical purposes. The antigens were presented by fusing them to the TasA protein without altering the morphology of the engineered cells (Vogt *et al*., 2018).

The most important trait of *B. subtilis* for heterologous protein production, its secretory capacity, has also received much attention as an avenue for improving protein yields. The Sec-pathway is the general protein secretion pathway and is most often used for heterologous protein secretion. The pathway consists of the SecA ATPase and the SecYEG pore complex (Karamanou *et al*., 1999; Brundage *et al*., 1990). Both SecA and SecYEG have been successfully engineered to improve protein expression (Mulder *et al*., 2013; Kakeshita *et al*., 2010). Another protein secretion pathway, Tat, has been used considerably less for protein secretion than Sec although it has the advantage of transporting fully folded proteins. This reduces the chance of the protein being degraded by extracellular proteases, thereby increasing protein yield. The Tat-pathway consists of the several different protein complexes: TatAd, TatAdCd, TatAy and TatAyCy, which can transport proteins of different sizes (Barnett *et al*., 2007; Barnett *et al*., 2008). Although *B. subtilis* has several other [ja] protein secretion pathways, these are rarely used for heterologous protein expression.

This review aims to summarize the molecular mechanisms of the Sec and Tat secretion pathways of *B. subtilis* and discuss how these mechanisms have been tweaked to increase expression and secretion of heterologous proteins. Discussion of the secretion pathways will focus on the translocation machinery, chaperones required for correct protein folding, and extracellular proteases (**Figure 1**). Heterologous protein expression has also been increased by engineering autolysins, prophages and cannibalism factors, but these will not be discussed in this review due to time constraints.

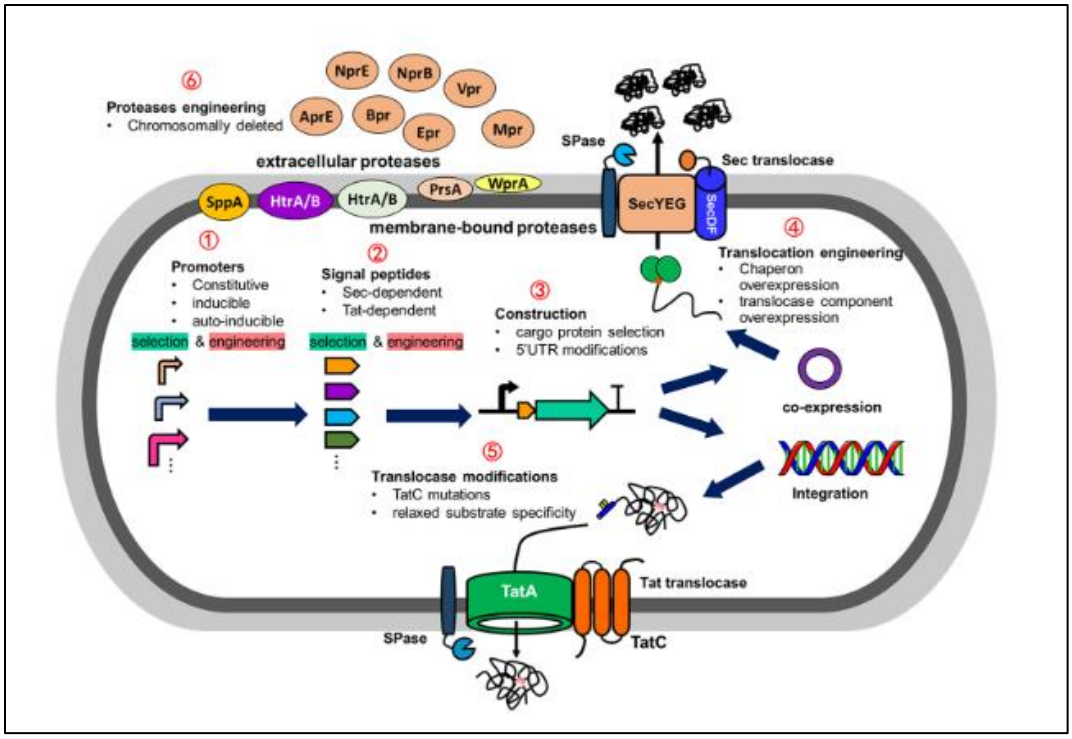

Figure 1. An overview of engineering targets for improving heterologous protein expression in *B. subtilis*. This review focuses on signal peptides (2), translocation engineering (4), translocase modifications (5) and protease engineering (6). From Cui *et al*. (2018).

Chapter 1. The Sec pathway

1.1 From ribosome to the translocation machinery

Much knowledge about the molecular mechanisms of protein secretion is obtained from studies on *E. coli*. The proteins forming the Sec-translocation machinery of *B. subtilis* are similar to those in *E. coli* although it lacks a SecB protein. The Sec pathway translocates unfolded proteins which are folded extracellularly with the help of chaperones. The translocation process starts at the ribosome where the protein to be secreted is translated and from which it emerges as a preprotein. After transcription and translation of the genes of interest the preprotein must be guided to and through the Sec machinery to be translocated to the extracellular medium. The Sec pathway is used by *B. subtilis* for most protein secretion. Preproteins are tagged for secretion through the use of a signal peptide that is present at their N-terminus. Most secretion signal peptides of *B. subtilis* are made up of a positively charged N-domain, a hydrophobic core domain and a polar C-domain that ends with the consensus amino-acid sequence AXA, where X can be any residue (von Heijne, 1985). The signal peptide is cleaved at the AXA site after translocation into the extracellular medium by type I signal peptidases of the Sip family (**Figure 2**) (Tjalsma *et al*., 1998). This process turns the preprotein into the mature secreted protein. The signal peptide is the first part of the protein emerging from the ribosome and can be bound by the SecA

protein to be guided to the rest of the Sec translocation machinery. Besides protein secretion, the Sec translocation pathway is also used for insertion of membrane proteins. Membrane proteins do have a signal sequence that is highly hydrophobic and is not cleaved by signal peptidase (von Heijne, 1985).

There are two modes of Sec translocation: co-translational and post-translational. Co-translational translocation is mostly used for membrane proteins and therefore less relevant to the production of heterologous proteins that are excreted into the extracellular medium. Post- translational translocation is used almost solely for secretory proteins and is therefore the most interesting for heterologous protein production.

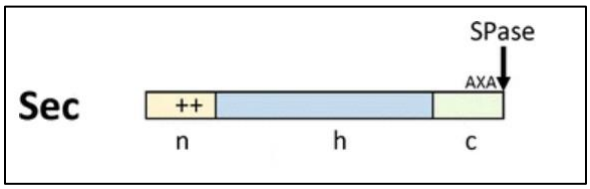

Figure 2. Sec signal peptide. The Sec signal peptide is divided into an N-domain, an H-domain and a C-domain. The N-domain is positively charged, the H-domain is hydrophobic and the C-domain is polar with an AXA cleavage site as the last three residues. From Freudl (2018).

Co-translational translocation is initiated by the signal recognition particle (SRP). The SRP consists of the Ffh and HBsu proteins and a small cytoplasmic RNA (scRNA) that functions as a scaffold. Ffh is a GTPase that can bind the scRNA to form a nucleoprotein that localizes to the cell membrane (Honda *et al*., 1993; Nakamura *et al*., 1994). HBsu is a histone-like protein that binds to the scRNA (Nakamura *et al*., 1999). This binding is specifically targeted to the secondary structure of the scRNA. During co-translational translocation the SRP guides the ribosome and the nascent preprotein together to the Sec machinery where the protein is translocated as it leaves the ribosome (**Figure 3A**). The SRP binds to the L23 protein of the ribosome, near the exit tunnel where the nascent polypeptide emerges, and binds to the signal peptide (Gu *et al*., 2003). The SRP complex has affinity for FtsY, a protein receptor that is present at the cell membrane, and guides the entire ribosome nascent chain complex (RNC) to this protein (Luirink *et al*., 1994). The FtsY protein associates with the Sec translocation pore (see below) to guide the nascent polypeptide through this pore. FtsY itself also has GTPase activity, which is required together with the GTPase activity of Ffh in the SRP to trigger the release of the ribosome from the FtsY-SRP complex (Bahari *et al*., 2007). The RNC then associates with the Sec translocation pore via the signal peptide of the nascent polypeptide to initiate its translocation. The conformational changes in the GTPase domains of FtsY and Ffh upon binding of the RNC are required for GTP hydrolysis and regulate the targeting of proteins to the membrane (Shan *et al*., 2017).

During post-translational translocation SecA binds the signal peptide of the preprotein and after translation has been completed, guides the preprotein to the rest of the Sec translocation machinery with the help of chaperones to keep the protein translation competent (**Figure 3A**). In Gram-negative bacteria the SecB protein functions as a chaperone that guides the Sec-transported protein from the ribosome to SecA. The latter subsequently transfers it to the rest of the Sec machinery for translocation (Randall *et al*., 2005). The Sec pathway of *B. subtilis* does not have a SecB protein (Van der Sluis & Driessen, 2006). The B. subtilis protein CsaA was suggested to fulfill the function of SecB as it is known to

bind to SecA (Müller *et al*., 2000b). Additionally, CsaA suppressed protein export defects in an *E. coli* strain lacking SecB and suppressed the heat sensitivity of *E. coli* strains lacking the intracellular chaperones DnaK, DnaJ or GrpE. This indicates that CsaA also acts as a chaperone, helping proteins to fold correctly (Müller *et al*., 2000a). Recently, *E. coli* SecA was found to interact with the L23 protein of the ribosome, near the exit tunnel where nascent polypeptides emerge, to guide the nascent protein to the Sec translocon. This suggests that SecA can bind to preproteins while they are being translated (Huber *et al*., 2011). Thus, contrary to earlier beliefs, the components of the post-translational pathway interact with the protein to be secreted during translation.

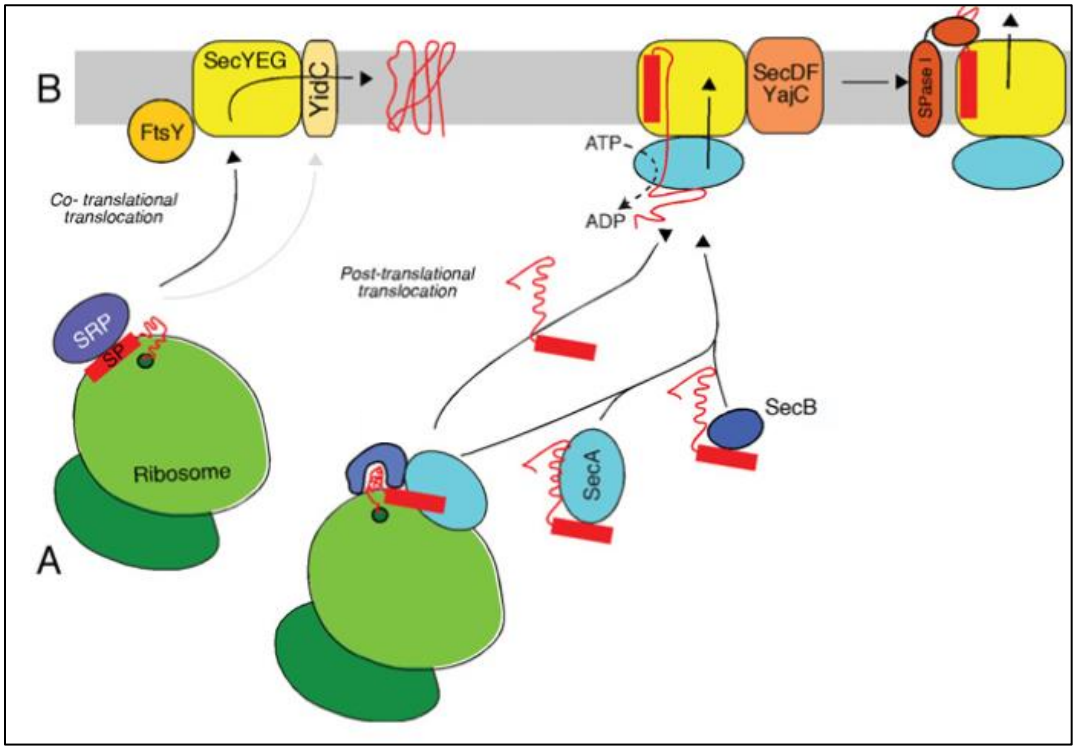

Figure 3. Post-translational and co-translational Sec translocation. (A) Targeting of the preprotein to the Sec translocation machinery. The left part shows co-translational translocation via SRP. The ribosome is guided to the Sec translocon through binding of SRP to FtsY. The right part shows post-translational translocation via SecA or SecB. In *B. subtilis* SecB does not exist and SecA functions to guide the preprotein to the Sec translocation machinery. (B) Translocation of the preprotein. The left part shows insertion of an integral membrane protein by the SecYEG translocation pore. The right part shows hydrolysis of ATP by SecA, which powers translocation of the preprotein through the SecYEG translocation pore with the help of SecDFYajC. After translocation the signal peptide is cleaved off by a type-I signal peptidase. SP: signal peptide. Adapted from Chatzi *et al*. (2013).

1.2 Components of the Sec translocation machinery

The core *B. subtilis* Sec translocation machinery consists of three proteins and an additional four non-essential proteins. Besides targeting preproteins to the Sec translocation machinery, SecA functions as

an ATPase that couples ATP hydrolysis to the translocation of proteins (**Figure 3B**) (Karamanou *et al*., 1999). Binding of preproteins to the protein binding domain of SecA is required for the ATPase function (Karamanou *et al*., 2007). Electron cryo-microscopy of *E. coli* Sec proteins has elucidated the structure of the Sec translocon in ever greater detail, thereby providing clues to the mechanism behind Sec protein translocation (Ma *et al*., 2019). SecA has two nucleotide binding domains (NBD) at its N-terminus. NBD1 and NBD2 are required for the binding and hydrolysis of ATP (Sianidis *et al*., 2001; Hunt *et al*., 2002). SecA also contains a two-helix finger (THF) domain and a clamp domain. The THF binds the preprotein and reaches through the translocation pore while the clamp positions the preprotein above the pore (Erlandson *et al*., 2008; Banerjee *et al*., 2017). SecA can be inserted in the membrane and subsequently deinserted. This cycle is what drives preprotein translocation. Preprotein binding to membrane-inserted SecA leads to ATP hydrolysis and deinsertion of SecA. The preprotein is then released from SecA, having translocated 20-30 amino acid residues and then SecA reinserts into the membrane, resetting the cycle (Economou & Wickner, 1994). The proton motive force greatly speeds up the translocation of preproteins during the part of the cycle where the protein and ATP are not bound to SecA (Schiebel *et al*., 1991). For a long time there were two competing models of the mechanism by which SecA translocates preproteins. The Brownian ratchet model posited that the binding of ATP to SecA opens the translocation pore. The preprotein can then diffuse through the channel and subsequent ATP hydrolysis closes the channel and prevents the preprotein from diffusing back (Allen *et al*., 2016). The power stroke model, also called push-and-slide, posits that ATP binding causes the THF to push the preprotein through the channel. Subsequent ATP hydrolysis retracts the finger to its original position while the clamp keeps the preprotein from sliding back (Bauer *et al*., 2014). Recent single-molecule FRET experiments have shown that the power stroke model is used by SecA (Catipovic *et al*., 2019).

Other than SecA, SecY and SecE are the only essential proteins of the Sec translation machinery (Brundage *et al*., 1990). SecY and SecE form a membrane pore complex with SecG and together translocate the protein with the energy provided by SecA. SecY and SecE are integral membrane proteins with 10 and 1 transmembrane domain(s), respectively (Jeong *et al*., 1993; Nakamura *et al*., 1990). Electron cryo-microscopy experiments have shown that the SecY protein forms an hourglass-shaped pore that contains a constricting ring, a plug and a lateral gate (van den Berg *et al*., 2004; Egea & Stroud, 2010). The ring and the plug prevent small solutes from leaking out when there is no polypeptide in the channel (Li *et al*., 2007; Park & Rapoport, 2011). Preventing membrane leakage through the Sec pore is so important that other segments of SecY will form a plug if the original plug is deleted (Li *et al*., 2007). The lateral gate opens during co-translational translocation to insert proteins into the membrane (Stroud & Egea, 2010). The SecY protein was shown to be vulnerable to breakdown by the membrane-bound protease FtsH. The SecE protein binds to SecY and thereby prevents the degradation of SecY by FtsH in *E. coli* (Taura *et al*., 1993). Since FtsH is also present in *B. subtilis* as well it is expected that *B. subtilis* SecE has the same role in Sec translocation (Lysenko *et al*., 1997). SecY and SecA form punctate clusters on the cell membrane, which are arranged in larger helical patterns. SecA localization is directed by anionic membrane lipids (Campo *et al*., 2004). SecY localization on the other hand, is directed by the interaction between SecY and FloT. FloT is a flotillin, a protein which inserts into the cell membrane with hydrophobic loops and oligomerizes to form microdomains (Bach & Bramkamp, 2013)

The other components of the *B. subtilis* Sec translation machinery, SecD, SecF, SecG and YajC,

are not essential but do improve the rates of protein translocation by SecY, SecE and SecA. SecG is an integral membrane protein that can adopt two topologies. The transition of SecG between these topologies has a low energetic cost, is coupled to the membrane insertion cycle of SecA (Nishiyama *et al*., 1996) and decreases the energetic barrier of SecA insertion and deinsertion. This energetic barrier is most difficult to overcome when little thermal energy is present and *secG* mutants are therefore sensitive to low temperatures. A SecA mutant, *secA36*, designed to suppress a mutation in SecY that prevents SecA insertion, did not exhibit reduced viability when exposed to a low temperature (20 ^{o}C) and SecG was knocked out. SecA36 of this mutant allows for membrane insertion in the absence of SecG, whereas the wild type SecA does not. These results show that SecG increases the rate of SecA insertion (Matsumoto *et al*., 1998). Additionally, when ATP concentrations are low, SecG is essential for protein secretion at 37 ^{o}C when combined with either wild type SecA or SecA36.

SecD and SecF are separate proteins in many prokaryotes but are fused together in *B. subtilis* (Bolhuis *et al*., 1998). SecDF is an integral membrane protein and was shown to increase the secretion capacity of the cell. SecDF was also hypothesized to release mature proteins from the membrane into the extracellular medium, because *E. coli* SecD is known to do so (Matsuyama *et al*., 1993). However, SecDF depletion did not lead to an accumulation of mature proteins on the membrane of *B. subtilis*, indicating that release of mature proteins is not a function of *B. subtilis* SecDF (Bolhuis *et al*., 1998). The exact mechanism through which SecDF increases protein secretion capacity is not entirely clear. SecDF was shown to have structural similarities to secondary solute transporters, which led to the hypothesis that it clears misfolded proteins that jam the Sec translocon and accumulate during high protein secretion (Bolhuis *et al*., 1998). Another hypothesis assumes that SecDF is responsible for stimulating protein translocation via the proton motive force. This supposition is based on the finding that *E. coli* SecD and SecF are required to maintain the proton motive force in inverted membrane vesicles (Arkowitz & Wickner, 1994). The third hypothesis is that SecDF is required for assembly of the translocation complex. Recently crystal structures of SecDF have lent weight to the idea that SecDF uses the proton motive force to stimulate protein translocation (Tsukazaki *et al*., 2011). However, the mechanism through which this occurs has to date not been definitively established (Tsukazaki, 2018)

The protein YajC of *B. subtilis* forms a complex with SecDF but its function has not been clarified yet (Taura *et al*., 1994). Thus far, research has focused on the function of SecDFYajC but not on the individual function of YajC (Schulze *et al*., 2014; Komar *et al*., 2016). It was shown to be dispensable in the wild type translocation machinery but it could suppress the secretion defects caused by a mutation in *secY*, *secY^{d}1* (Taura *et al*., 1994). This suggests that YajC interacts with SecY but the interaction is not essential in most cases. The SecDFYacJ complex interacts with the SecYEG complex to form a hexatrimeric holoenzyme SecYEGDFYajC. SecDFYajC was shown to have a function similar to SecG, facilitating the insertion of SecA into the cell membrane. In experiments measuring the translocation of the Sec-targeted preprotein proOmpA out of inverted membrane vesicles, the translocation rate was not dependent on the presence of SecDFYacJ in strains expressing SecG. However, in *ΔsecG* strains the presence of SecDFYacJ increased the translocation rate of proOmpA (Duong & Wickner, 1997).

Chapter 2. The Tat pathway

2.1 From the ribosome to the translocation machinery

The Tat pathway is has a narrower substrate range but has received significant attention for biotechnological purposes because it transports folded preproteins. Transporting folded preproteins instead of unfolded proteins, the latter of which are then folded in the extracellular medium, decreases the risk of protein degradation by extracellular proteases (DeLisa *et al.*, 2003). This advantageous trait of the Tat secretion pathway compensates for one of its disadvantages, namely its narrow substrate range. Since the preproteins fold on their own in the cytosol the Tat translocation machinery does not interact with the ribosome to guide nascent polypeptide chains to the translocation pore, such as happens in the Sec pathway (see above). Instead, the fully folded preproteins interact with a docking complex on the membrane through their signal peptide and are then transferred through the translocation pore into the extracellular medium. The signal peptide is then cleaved by a signal peptidase outside the cell (**Figure 4**).

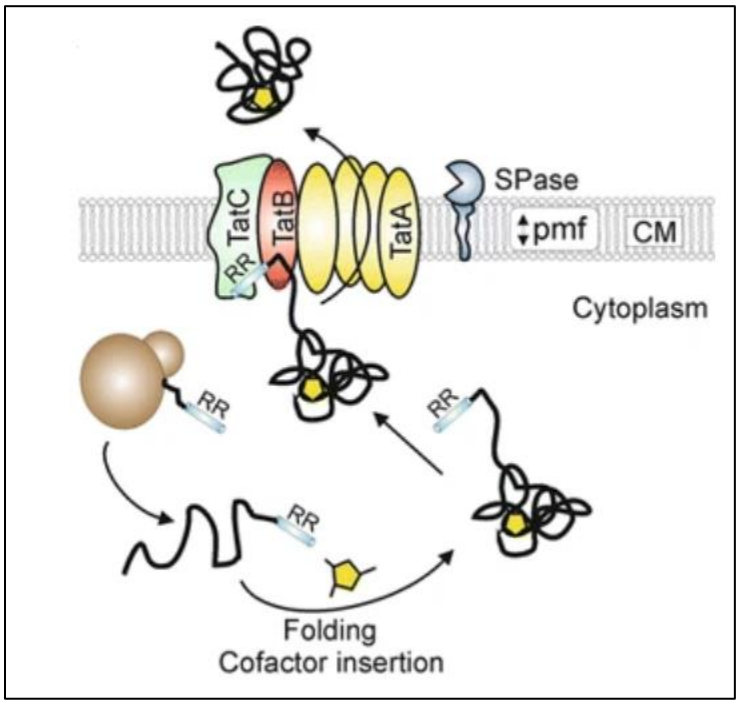

Figure 4. Protein translocation via the Tat pathway. Preproteins are folded before translocation and contain a twin arginine motif. The Tat signal peptide is recognized by TatC and TatB, which together transfer the preprotein to the translocation pore made up of a variable number of TatA subunits. In *B. subtilis* the function of TatB is fulfilled by the TatA protein. From Freudl (2018).

The Tat pathway derives its name from the twin arginine motif that defines the signal peptide for the Tat pathway. The signal peptide of preproteins that are secreted via the Tat pathway has a similar overall structure with a positively charged N-terminal domain, a hydrophobic core and a polar C-terminal domain with an AXA consensus cleavage site. The twin arginine motif is located in the N-terminal domain of the signal peptide and has the consensus sequence (S/T)-R-R-x-F-L-K, where the x is a hydrophobic residue (Berks, 1996). The arginine residues are always present in this motif while the

other residues are present in more than 50% of Tat signal peptides (**Figure 5**). Additionally, the hydrophobic core of the Tat signal peptide sequence is less hydrophobic than that of the Sec signal peptide (Cristobál *et al*. 1999). Moreover, positive residues in the c-domain are also required to avoid targeting of Tat substrates to the Sec pathway (Blaudeck *et al*., 2003). However, there are also cases of Tat-secreted proteins without a Tat signal peptide but instead hitchhike with other proteins that do have the Tat signal peptide (Rodrigue *et al*., 1999)

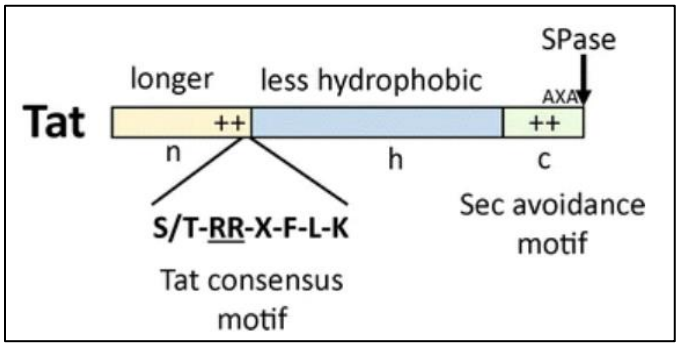

Figure 5. The Tat signal peptide. The Tat signal peptide has a structure similar to that of the Sec signal peptide, with an N-domain, hydrophobic core and C-domain. Differences between the two are pinpointed in the figure. The Tat signal sequence prevents protein translocation via the Sec pathway with a twin arginine motif, the presence of a less hydrophobic core region and a positively charged C-domain. From Freudl (2018).

2.2 Components of the Tat translocation machinery

The Tat pathway is conserved in Gram-negative bacteria, Gram-positive bacteria and the thylakoids of chloroplasts in plants. The *B. subtilis* Tat pathway consists of three different versions of the TatA protein and two vesions of TatC (**Figure 6B**). This is significantly different from the Tat pathway of Gram-negative bacteria and the plant thylakoids, which contain a TatA-like TatB protein that is involved in substrate recognition. Gram-negative bacterial and thylakoidal Tat-translocation machinery also contains only one type of TatA and TatC (**Figure 6A**). *E. coli* and thylakoid TatB and TatC form a docking complex which can recognize the twin arginine motif of the Tat signal peptides. After docking TatB recruits a varying number of TatA proteins which form a translocation pore. This produces a fully functional TatABC translocase (Bolhuis *et al*., 2001). The TatA proteins of *B. subtilis* can fulfill the functions of both TatA and TatB from Gram-negative and thylakoidal Tat translocase. This was first proposed based on the finding that the *B. subtilis* Tat-translocon can function without TatB and later confirmed by showing that *B. subtilis* TatAd can complement *E. coli* mutants without TatA or TatB (Jongbloed *et al*., 2004; Barnett *et al*., 2008). After the translocase is assembled *E. coli* and thylakoid TatB transfers the preprotein from the docking complex to the translocation pore. This process requires the proton motive force and is the rate limiting step of Tat translocation (Alami *et al*., 2003; Whitaker *et al*., 2012). The Tat translocation pathway is very different from that of all other protein translocation pathways in that it does not require the hydrolysis of GTP or ATP to function. Instead, in *E. coli* the electrical gradient across the cytoplasmic membrane was shown to be the driving force of Tat translocation (Bageshwar & Musser, 2007). In thylakoids both the electrical gradient and pH gradient

across the thylakoid membrane contribute to the translocation of proteins (Braun *et al*., 2007). In *E. coli* the proton motive force drives TatA oligomerization in combination with substrate recognition (Alcock *et al*., 2013). When the preprotein is transferred to the translocation pore, TatA controls the preprotein for proper folding and is capable of rejecting any protein that is misfolded (Matos *et al*., 2008). Properly folded preproteins are then translocated to the extracellular medium after which the signal peptide is cleaved a signal peptidase.

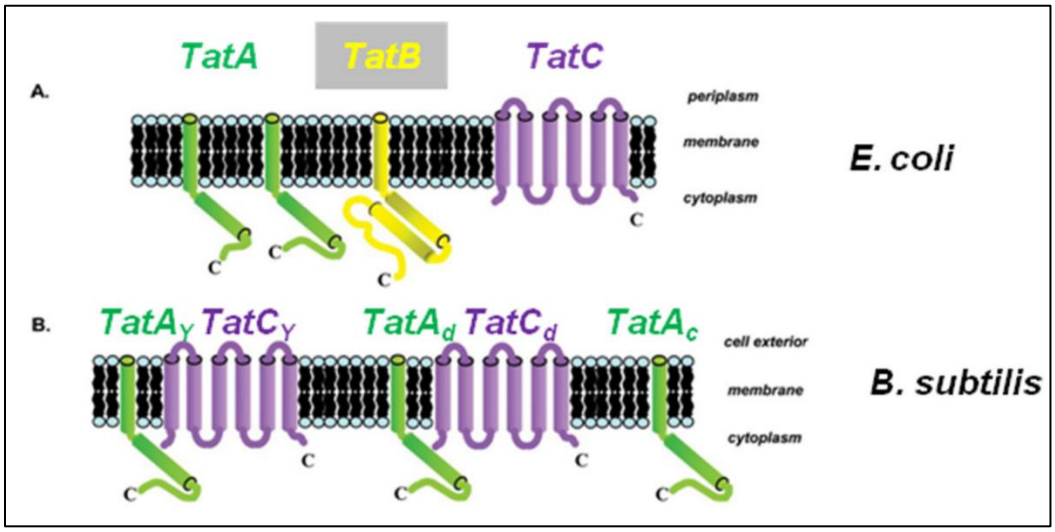

Figure 6. The Tat proteins of *E. coli* and *B. subtilis*. *E. coli* contains TatC, TatA and TatA-like TatB proteins. *B. subtilis* has three TatA protein variants and two TatC protein versions. TatAy and TatCy, and TatAd and TatCd form separate TatAC translocons. Adapted from Sargent (2007).

The three TatA proteins of *B.* subtilis are TatAd, TatAy and TatAc, and the two TatC proteins are TatCd and TatCy. TatAd and TatCd associate to form the TatAdCd complex while TatAy and TatCy form the TatAyCy complex (**Figure 6B**). Only the TatAdCd complex was shown to be essential for expression of Tat-preprotein PhoD, indicating that TatAdCd and TatAyCy have different substrates (Jongbloed *et al*., 2000). The TatAdCd complex has a narrower range of substrates than TatAyCy. However, overexpressed TatAd is capable of replacing TatAy and overexpressed TatAdCd shows an increased acceptance of many Tat proteins (Eijlander *et al*., 2009b). TatAd and TatAy also exist as homo-oligomeric complexes. The TatAdCd complex has a size of 230 kDa while the oligomeric TatAd complex has a size of 270 kDa. These complexes have discrete sizes, which differs from the *E. coli* TatABC complex as that can adopt a wide range of sizes which depends on the number of TatA subunits that form the translocation pore (Barnett *et al*., 2007; Gohlke *et al*., 2005). The variation in the sizes of the Tat complexes allows the Tat translocation machinery to secrete proteins of various sizes. The TatAyCy complex is smaller than TatAdCd, which has a size of 200 kDa (Barnett *et al*., 2008). Similar to TatAdCd, the TatAyCy complex is relatively homogenous in size. Interestingly, although TatAdCd and TatAyCy of *B. subtilis* have different morphologies from that of *E. coli* TatABC the three complexes are able to secrete similar proteins (Barnett *et al*., 2008). This suggests that the TatAC complexes of *B. subtilis* can alter their size to accommodate proteins of different sizes and are less rigid than originally thought.

The function of the TatAc protein was elucidated more recently. TatAc was shown to partially

alleviate growth defects of strains with mutated TatAy proteins on low salt media. When TatAc was fivefold overexpressed it was capable of fully compensating for the reduced translocation caused by TatAy-P2D, -P21A, -A31G and -G32A (Goosens *et al*., 2015). The P2D mutation destabilizes the TatAy protein (van der Ploeg *et al.,* 2011). Yeast two hybrid assays showed that the P21A, A31G and G32A mutations reduced the ability of TatAy to self-interact. This hampers oligomerization and the formation of the translocation pore. However, TatAc could not form fully functional translocons with either TatCy or TatCd, which indicates that TatAc has a supporting role in the Tat pathway. Wild type TatAy can form both a docking complex for the Tat-translocated substrate and a pore complex for translocation. The mutations in TatAy impaired its pore forming capabilities, which could be compensated for by TatAc. TatAc however is not capable of forming a docking complex with TatCy or TatCd. This situation is reminiscent of the TatA and TatB proteins of *E. coli*, where TatAy fulfills the role of the TatB protein and TatAc fulfills that of the TatA protein. It is therefore hypothesized that the TatAc protein represents an intermediate evolutionary state towards differentiation of the TatA protein into two distinct Tat proteins (Goosens *et al*., 2015). Another study however, found that the TatAc protein forms functional translocation complexes with both TatCd and TatCy. Expression of TatAc and TatCd or TatCy in an *E. coli* mutant lacking all endogenous Tat genes led to the secretion of the Tat proteins TorA, AmiA and AmiC, indicating that the TatAcCy and TatAcCd complexes were fully functional (Monteferrante *et al*., 2012).

Chapter 3. Chaperones

3.1 Intracellular chaperones

Intracellular chaperones are important to the secretion process because they keep preproteins in a secretion-competent state and ensure proper folding of expressed proteins. For this reason many studies have used these chaperones to improve the protein secretion capacity of *B. subtilis*. *B. subtilis* has two main operons that encode intracellular chaperones: the *dnaK* operon and the *groE* operon. The genes of the *dnaK* and *groE* operons encode class-I heat shock proteins and are upregulated to combat heat stress by preventing the misfolding of proteins. This is reflected in the fact that the DnaK chaperone machinery is essential for survival at high temperatures (Mogk *et al*., 1997). The *dnaK* operon contains the *hrcA* gene, which encodes a protein that regulates *dnaK* and *groE* transcription; the *grpE*, *dnaK* and *dnaJ* genes, which encode the intracellular chaperones; and three uncharacterized ORFs (**Figure 7**).

The DnaK protein is the main chaperone and is assisted by DnaJ and GrpE (Georgopoulos, 1992). Knowledge about the molecular mechanisms of DnaK and GroE chaperone function mostly comes from studies done in *E. coli*. *E. coli* DnaK contains an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain which are linked by a hydrophobic linker (Bertelsen *et al*., 2009). The binding and folding of substrates by DnaK is regulated by ATP. Recently, it was shown that ADP-bound DnaK assists protein folding by holding the substrate protein in its unfolded state. When the ADP is exchanged for ATP the substrate is released which allows the protein to refold (Winardhi *et al*., 2018). DnaJ and GrpE modulate the binding of ATP and ADP to DnaK, thereby regulating protein folding by

DnaK. Phosphorylation of DnaK is crucial for the interaction between it and its cochaperones and the Y601 phosphorylation site was specifically shown to be important (Shi *et al*., 2016) Upon binding of ATP by the NBD the NBD and SBD both undergo conformational changes that reduce the affinity of DnaK for its substrates (Meinhold *et al*., 2019). Binding of a substrate to the SBD reverses this conformational change and thereby increases the ATPase activity of the NBD (Swain *et al*., 2007). DnaJ further stimulates ATP hydrolysis, thereby regulating the conversion of DnaK from its low affinity conformation to the high affinity conformation. DnaJ can only stimulate ATP hydrolysis of DnaK when a substrate is bound to the SBD of DnaK (Laufen *et al*., 1999). DnaJ has an N-terminal J domain that contains a positively charged helix with which it binds to a negatively charged loop in the DnaK NBD. Through this interaction DnaJ can regulate the ATPase activity of DnaK (Ahmad *et al*., 2011). GrpE stimulates the release of ADP from DnaK, which returns DnaK to its low affinity conformation (Mally & Witt, 2001). This causes the substrate to be released from DnaK (Pakschies *et al*., 1997). GrpE binds to DnaK as a dimer, with one of the monomers contributing most of the residues that interact with DnaK. One GrpE subunit binds to the NBD of DnaK and opens up the structure, thereby disrupting ATP binding. The N-terminal helices of the two GrpE subunits extend to the SBD of DnaK and releases the bound protein from this domain (Harrison *et al*., 1997).

The *groE* operon contains the *groES* and *groEL* genes, which encode intracellular chaperones. GroEL is the main chaperone and is assisted by GroES (Georgopoulos, 1992). *E. coli* GroEL forms a tetradecameric complex that consists of two identical heptameric rings (Braig *et al*., 1994). GroEL binds misfolded proteins by binding outwards facing hydrophobic residues with its own hydrophobic binding sites (Lin *et al*., 1995; Fenton *et al*., 1994). The ring binding the substrate is called the *cis*-ring while the ring is called the *trans*-ring. Similar to DnaK, the folding activity of GroEL is also regulated by the binding of ATP and the GroES cochaperone. GroES forms a heptameric ring complex which can form a cap on the GroEL cylinder (Hunt *et al*., 1996). 7 ATP molecules bind to GroEL which recruits GroES and subsequent ATP hydrolysis creates a stable GroEL-7ADP-GroES complex. This expands the hydrophilic cavity of GroEL and forces the bound substrate into the cavity where it can fold in a proteolytically protected environment (Martin *et al*., 1993; Mayhew *et al*., 1996; Weissman *et al*., 1995). The GroES and substrate are released from the *cis*-ring by binding of ATP to the *trans*-ring. This mechanism leads to protein folding on alternating sides of the GroEL cylinder (Rye *et al*., 1999). Recently, the reaction kinetics of GroEL-GroES cycling were further elucidated through atomic force microscopy. This study revealed that the dynamics of GroE folding are more complicated than originally thought (Noshiro & Ando, 2018). However, due to time constraints these dynamics cannot be discussed.

The *B. subtilis dnaK* and *groE* operons are regulated by a σ^A promoter and an operator, CIRCE. CIRCE consists of two 9 bp inverted repeats that are separated by a 9 bp spacer (Zuber & Schumann, 1994). HrcA contains a helix-turn-helix motif with which it can bind to CIRCE and repress transcription of the *dnaK* and *groE* operons (Yuan & Wong, 1995; Wiegert & Schumann, 2003). HrcA is inactive after translation and GroE modulates the activity of HrcA by folding it into an active conformation. HrcA can then bind to the CIRCE element but when it is released it adopts its inactive conformation again (Schumann, 2003). Misfolded proteins compete with HrcA for binding to the GroE chaperone. An increase in misfolded proteins therefore inactive HrcA, which causes non-functional HrcA aggregates. This subsequently prevents HrcA from repressing *dnaK* and *groE* operon transcription, thereby

increasing the amount of intracellular chaperones to alleviate the heat stress that caused the increase in misfolded proteins (Mogk *et al*., 1997).

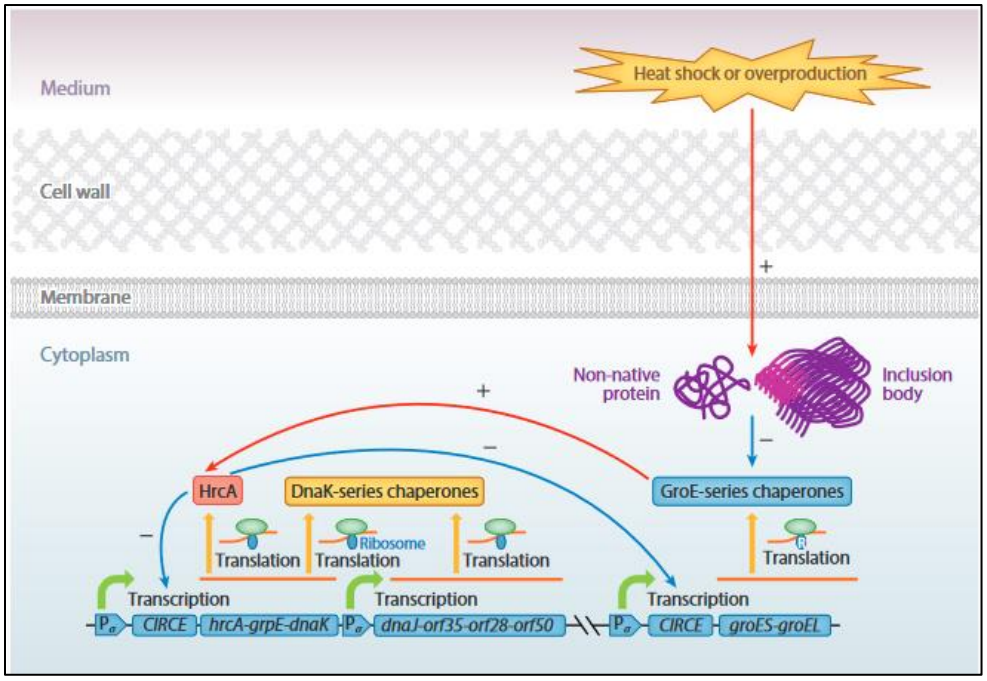

Figure 7. The *dnaK* and *groE* operons and their regulation. The HrcA protein suppresses transcription of both the *dnaK* and *groE* operons. Unbound GroE chaperone enhances HrcA function, thereby lowering intracellular chaperone levels. When GroE chaperone molecules are bound by misfolded proteins, HrcA function is downregulated, increasing the levels of intracellular chaperones. From Zhang *et al*. (2020).

3.2 Extracellular chaperones

Once a heterologous protein is secreted through a Tat-independent pathway it is still at risk of being degraded by proteases or misfolding. Extracellular chaperones and folding factors are present to increase the speed and accuracy of folding. The most canonical extracellular chaperone of *B. subtilis* is PrsA. PrsA is an extracellular lipoprotein bound to the outer leaflet of the plasma membrane and was found to be essential for protein secretion. Decreased levels of PrsA were correlated with decreased levels of exoproteins and overexpression of PrsA led to significantly increased exoprotein levels. These observations indicate that PrsA is a bottleneck in protein secretion and therefore attracted great interest as an optimization target for heterologous protein expression (Kontinen & Sarvas, 1993). *B. subtilis* mutants lacking PrsA were incapable of producing folded and functional subtilisin, *B. licheniformis* alkaline serine exoprotease (SubC) and *E. coli* alkaline phosphatase (PhoA). This shows that PrsA is a chaperone that is required for proper exoprotein folding (Jacobs *et al*., 1993). However, *B. subtilis* PrsA is essential for protein folding only in the presence of the cell wall. This indicates that it functions specifically to prevent interactions between secreted proteins and the cell wall (Wahlström *et al*., 2003). PrsA is also essential for the growth of *B. subtilis* because it assists the folding of four penicillin binding proteins (PBPs), which are involved in the synthesis of the cell wall (Hyyryläinen *et al.,* 2010). PrsA is a peptidyl-prolyl *cis-trans* isomerase (PPIase); PPIases catalyze the folding of *cis*-prolines,

which are often rate-limiting during protein folding (Schmid, 2001). PrsA belongs to the parvulin group of PPIases, many of which are involved in the protein maturation (Rahfeld *et al*., 1994; Behrens-Kneip, 2010). PrsA has a PPIase domain flanked by a 128 residue N-terminal domain and a 70 residue C-terminal domain (Tossavainen *et al*., 2006). Although PrsA is classified as a PPIase, the PPIase domain is not essential for cell viability while the N- and C-terminal domains are (Vitikainen *et al*., 2004). PrsA must form a dimer to function and the N- and C-terminal domains facilitate this dimerization. Moreover, the composite NC domain creates a bowl-shaped crevice in the PrsA dimer. This crevice contains hydrophobic sites and has similar structure as known chaperones, suggesting that this NC domain is involved in the chaperone function of PrsA (Jakob *et al*., 2015). The PPIase and NC domains have separate functions but both have been shown to interact with secreted propeptides in NMR experiments (Jakob *et al*., 2015).

Chapter 4. Proteases and quality control

4.1 Extracellular proteases

A different strategy for reducing heterologous protein degradation in *B. subtilis* is to look at the extracellular proteases and quality control machinery. *B. subtilis* has a naturally high protease activity, which poses problems for heterologous protein production (Li *et al*., 2004). The earliest eight extracellular proteases that were characterized in *B. subtilis* were divided into two classes: serine proteases and metalloproteases (**Table 1**). NprE and AprE are responsible for the biggest share of proteolytic activity in *B. subtilis*, whereas the other proteins play lesser roles. NprE and AprE are regulated via a complex pathway involving multiple repressors that ensures they are only expressed in the post-exponential phase (Barbieri *et al*., 2016). Despite being responsible for only a minor share of proteolytic activity, WprA is a disproportionately large obstacle to heterologous protein production. It is the only protease of the eight listed in Table 1 that binds to the cell wall and can therefore have an outsized role in proteolysis of secreted proteins (Stephenson & Harwood, 1998). Its accumulation in the cell wall means that it is particularly apt to degrading unfolded proteins emerging from the translocation machinery.

Extracellular proteases do not only hydrolyze heterologous proteins, but are also involved in the control of autolysis. Autolysins are enzymes that break down the peptidoglycan of the bacterial cell wall (Vollmer *et al*., 2008). These autolysins are hydrolyzed by extracellular proteases, thereby limiting their activity and keeping cell wall breakdown in check. One study showed that NprE and AprE are responsible for reducing autolytic activity in *B. subtilis* while the other proteases had a negligible effect (Stephenson *et al*., 2002). Another study, however, revealed that the LytF autolysin is degraded by the proteases Epr and WprA (Yamamoto *et al*., 2003). The apparent contradiction between these studies might be explained by the fact that LytF is involved in cell separation. Therefore, the different levels of LytF do not translate into different levels of autolysis, as was measured by Stephenson *et al*. (2002). These results show that improving heterologous protein expression is more complex than knocking out all extracellular proteases, since increased autolysis lowers protein yields.

Table 1. The eight major extracellular proteases of *B. subtilis*. All proteases except WprA localize to the extracellular medium; WprA is bound to the cell wall.

Protein	Protease type	Reference(s)
NprE	Metalloprotease	Yang *et al*., 1984;
AprE	Serine protease	Stahl & Ferrari, 1984
Mpr	Metalloprotease	Rufo *et al*., 1990
Epr	Serine protease	Brückner *et al*., 1990
Bpf	Serine protease	Sloma *et al*., 1990; Wu *et al*., 1990
NprB	Metalloprotease	Tran *et al*., 1991
Vpr	Serine protease	Sloma *et al*., 1991
WprA	Serine protease	Margot & Karamata, 1996; Babé & Schmidt, 1998

4.2 Quality control proteases

Besides the eight main extracellular proteases *B. subtilis* has more proteases, of which the HtrA and HtrB proteases are the most relevant to heterologous protein secretion. HtrA and HtrB are classified as type-V heat shock proteins (Darmon *et al*., 2002). The HtrA and HtrB proteases are anchored to the outer leaflet of the cell membrane. However, HtrA has also been observed in an extracellular form (Antelmann *et al*., 2003). The extracellular protein lacks the first 96 N-terminal residues including the transmembrane domain of HtrA.

HtrA of *E. coli* was shown to function as a chaperone at low temperatures and as a protease at high temperatures (Spiess *et al*., 1999). *B. subtilis* HtrA also has chaperone activity, which was determined by showing that extracellular concentrations of the YqxI protein positively correlate with HtrA levels even when the protease function of HtrA is knocked out (Antelmann *et al*., 2003). The higher YxqI levels were not caused by an increase in transcription of the *yqxI* gene which indicated that they are post-translationally modulated by HtrA. Proteomic analysis of the extracellular environment of several protease deficient *B. subtilis* strains showed that 14 proteins were present in the exoproteome of the BRB08 strain, deficient for the eight proteases from **Table 1**, which were not present in the exoproteome of the BRB14 strain deficient in HtrA and HtrB and the eight aforementioned proteases (Krishnappa *et al*., 2013). This result implies that the 14 proteins require HtrA and/or HtrB to be folded correctly and points to a chaperone function of HtrA and HtrB. Additional functions of HtrA and HtrB are the cleaving and release of membrane bound lipoproteins and quality control of membrane proteins (Krishnappa *et al*., 2013).

HtrA and HtrB are regulated by the two component system CssS-CssR, which responds to heat and secretion stress (Darmon *et al*., 2002; Westers *et al*., 2006). CssS senses the accumulation of misfolded proteins at the cell membrane and responds with autophosphorylation (**Figure 8**). The Phosphorylated CssS then phosphorylates the cognate regulator, CssR. CssR~P subsequently alters the transcription *htrA*, *htrB*, and *CssRS* to remedy the secretion stress at the membrane. The *htrA*, *htrB* and *cssRS* operons are upregulated by activated CssR, which leads to a positive feedback loop that will increase the levels of HtrA and HtrB proteases until the secretion stress has been resolved (Hyyryläinen

et al., 2005; Hyyryläinen *et al*., 2008). CssS, HtrA and HtrB are distributed in foci across the cell membrane. Curiously though, HtrA and HtrB do not localize to the same areas as CssS (Noone *et al*., 2012). This result implies that HtrA and HtrB do not go to the location where secretion stress is detected, but solve the problem in another location on the cytoplasmic membrane. To explain these observations it has been hypothesized that *B. subtilis* HtrA and HtrB localize to the same regions in the cytoplasmic membrane as the secretion machinery to deal with secretion stress. Such a situation has been observed in *S. pyogenes* in which the existence of an ExPortal region has been proposed (Rosch & Caparon, 2005). In *S. pneumonia*, HtrA localizes to the septum and equatorial regions of dividing cells, regions where cell division and cell wall synthesis takes place. This localization is dependent on the presence of the anionic membrane lipid cardiolipin (Tsui *et al*., 2011) Moreover, many *B. subtilis* proteins are known to localize into microdomains (Lucena *et al*., 2018). For instance the membrane of *B. subtilis* contains microdomains that are dedicated to protein secretion (see Chapter 1). These microdomains get their structure from flotillins, proteins which have hydrophobic loops that insert into the membrane and which oligomerize to form lipid rafts (Bach & Bramkamp, 2013).

In addition to regulation by CssRS, HtrB is known to be autoregulated, reducing its own expression when high levels of HtrB are present. HtrA and HtrB also regulate each other's expression. HtrA knockout leads to HtrB overexpression and vice versa (Noone *et al*., 2001). Removal of one of the proteases does not lead to secretion stress because the other compensates for this loss, showing that HtrA and HtrB have overlapping functions.

The ability of the quality control proteases to alleviate secretion stress implies that they could improve cell functioning upon protein overproduction and thus increase heterologous protein expression. Moreover, the chaperone functions of HtrA and HtrB could improve protein folding and increase the yield of heterologous proteins. This makes these proteases a less desirable target for deletion than the eight extracellular proteases. Finally, more extracellular proteases than the ones discussed here have been predicted in *B. subtilis* but not functionally identified yet (Krishnappa *et al*., 2013). More knowledge about these proteases could further help optimize *B. subtilis* as an expression host for heterologous proteins.

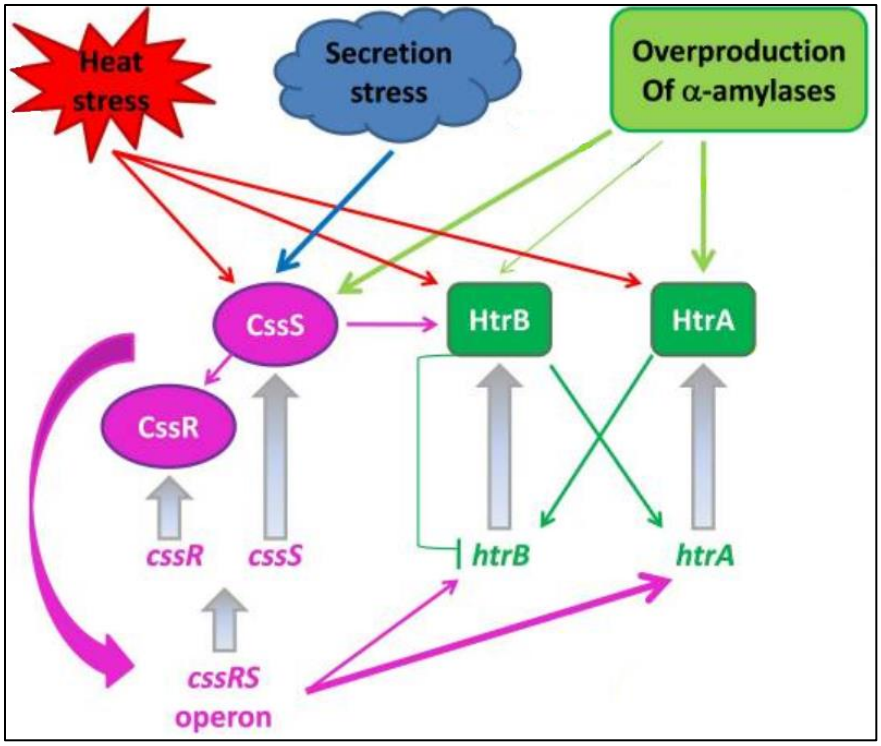

Figure 8. Regulation of quality control proteases HtrA and HtrB. HtrA and HtrB are regulated by the CssRS two component system. CssRS also regulates its own expression leading to a positive feedback loop that quickly magnifies its response to heat or secretion stress. HtrA and HtrB also cross-regulate each other, leading to overexpression of one protease if the gene of the other is deleted. Adapted from Yan & Wu (2019).

Chapter 5. Optimization of secretion pathways

Each part of the secretion pathways described in Chapters 1-4 has been engineered to optimize protein expression with varying levels of success. The knowledge gained over the past decades has fueled many achievements in the optimization of heterologous protein secretion. This final chapter discusses the work performed on optimization of each of the components of the secretion pathways of *B. subtilis*. Moreover, accomplishments are listed in **Table 2**. In addition to the studies listed in **Table 2**, many more studies have accomplished an increase in heterologous protein expression through similar engineering strategies. However, these cannot be discussed due to time constraints.

Table 2. Improvements made to *B. subtilis* for heterologous protein expression.

Expressed protein	Modification(s)	Secretion effect	Reference
Fusarium solani pisi cutinase	Fusion of Epr signal peptide	Extracellular activity of 4.67 U/mL	Brockmeier *et al*., 2006
B. amiloliquefaciens α-amylase	Overexpression of SecYEG	300% increase	Mulder *et al*., 2013
E. coli PhoA	Co-expression of chimeric SecA and *E. coli* SecB	60% increase	Diao *et al*., 2012
E. coli PhoA and *B. licheniformis* α-amylase	Fusion with non-canonically secreted RDPE protein	Extracellular activity of 870 U/mL; 63 U/mL	Chen *et al*., 2016
Anti-digoxin single chain antibody	Inactivation of *hrcA* and *wprA*, PrsA overexpression	250% increase	Wu *et al*., 1998
Recombinant human FGF21	Overexpression of DnaK	987% increase	Li *et al*., 2019
B. amiloliquefaciens α-amylase and *B. licheniformis* subtilisin	Overexpression of PrsA	250% increase; 200% increase	Kontinen & Sarvas, 1993
Streptococcus pyogenes pneumolysin	Overexpression of PrsA	150% increase	Vitikainen *et al*., 2005
Five heterologous α-amylases	Expression of cognate heterologous PrsA	Up to 150% increase	Quesada-Ganuza *et al*., 2019
B. pumilus γ-glutamyltranspeptidase	Overexpression of PrsA	100% increase	Yang *et al*., 2019
B. anthracis protective antigen	Overexpression of PrsA	250% increase	Williams *et al*., 2003
B. licheniformis α-amylase and *Geobacillus stearothermophilus* α-amylase	Overexpression of DnaK and PrsA	700% increase; 1200% increase	Chen *et al*., 2015
B. clausii ManA	Deletion of *nprE, aprE, mpr, epr, bpf* and *nprB*	Extracellular activity of 6041 U/mL	Zhou *et al*., 2018
Staphylococcus aureus staphylokinase	Deletion of *nprE, aprE, mpr, epr, bpf, nprB* and *vpr*	Extracellular concentration of 337 mg/L	Ye *et al*., 1999
Clostridium thermocellum XynX	Deletion of *nprE, aprE, mpr, epr, bpf, nprB, vpr* and *wprA*	Extracellular activity of 8.46 U/mL	Phuong *et al*., 2012
B. licheniformis α-amylase and *Geobacillus stearothermophilus* α-amylase	Inactivation of *ccsR*	50% decrease; 50% decrease	Vitikaine *et al*., 2005
B. naganoensis PUL	Deletion of *nprE, aprE, mpr, epr, bpf, nprB, vpr, wprA, srfC* and *spoIIAC* in the ATCC 6051 strain	48% increase	Liu *et al*., 2018

5.1 Optimization of the Sec pathway for protein secretion

The Sec pathway is the most studied secretion pathway and as such, most attempts to optimize the secretion capacity of *B. subtilis* have been made on this system. All parts of the Sec pathway described in Chapter 1 have been altered independently or in combination in order to improve heterologous protein expression.

One strategy for improving the Sec translocation pathway to increase heterologous protein expression has been to find signal peptides that stimulate protein secretion. One study screened all naturally occurring signal peptides of the Sec pathway in *B. subtilis* and fused these to a cytoplasmic esterase and a cutinase from *Fusarium solani pisi*. The amount of these heterologous proteins in the extracellular medium showed the efficacy of all signal peptides. The signal peptides that resulted in high protein secretion of the esterase were poorly capable of directing the secretion of the cutinase and vice versa (Brockmeier *et al*., 2006). This shows that for different heterologous proteins different signal peptides result in optimal secretion. This hinders the engineering of highly expressed heterologous proteins because the lengthy screening process must be performed for each heterologous protein.

Numerous alterations have also been made to the proteins of the Sec pathway in *B. subtilis* to improve protein secretion. Overexpression of proteins can lead to an overload of protein that cannot be translocated due to the lack of a large number of translocons. Because of this engineering stronger promoters can only increase protein yield by a limited amount without also making more translocons available. The secretion of *Bacillus amiloliquefaciens* α-amylase (AmyQ) was increased by 300% by overexpression of the SecYEG protein complex through an artificial secYEG operon (Mulder *et al*., 2013). Other teams have looked at the SecA protein as a way of increasing the protein secretion capacity of *B. subtilis*. By expressing a chimeric SecA in *B. subtilis* with the 32 C-terminal residues replaced by the corresponding residues of *E. coli*, the SecB protein from *E. coli* could be co-expressed. This hybrid Sec translocon increased secretion of *E. coli* PhoA by 60% (Diao *et al*., 2012). In another study the 61 C-terminal residues of SecA were deleted. This deletion increased the secretion of alkaliphilic *B. subtilis* sp. thermostable alkaline cellulose by 83% and the secretion of human interferon α by 220%.

5.2 Optimization of the Tat pathway for protein secretion

Optimization of the Tat pathway has thus far proven less fruitful than the optimization of the Sec pathway. This is due to the fact that the Sec pathway is the most studied pathway of *B. subtilis* and most proteins are naturally translocated by the Sec pathway. However, Tat has seen growing interest for biotechnological purposes and thus it is expected that achievements similar to those with the Sec pathway will happen in the future.

Utilization of the Tat pathway has been attempted by fusing Tat signal peptides to the N-termini of heterologous proteins. However, this way of using the Tat pathway is hindered by the lack of characterized signal peptides. The best characterized and most widely used signal peptides are PhoD, YwbN and QcrA (Palmer & Berks, 2012). Another obstacle is the fact that there is no signal peptide that optimizes the secretion of every protein. Because of this engineers must go through the lengthy process of finding a good signal peptide for every new heterologous protein that will be secreted. The most common way to do this is through screening of extensive signal peptide libraries. The Tat signal peptide YwbN was shown to direct secretion of subtilisin through the Tat translocation pathway, but directed α-amylase secretion through a Tat-independent pathway (Kolkman *et al*., 2008). In another study the

PhoD Tat signal peptide also directed GFP secretion through a Tat-independent pathway (Snyder *et al*., 2014). These studies show that the effect of fusing a Tat signal peptide to the desired heterologous protein cannot be predicted yet.

Improving the Tat translocation pathway to secrete more heterologous proteins has been less fruitful than the improvement of the Sec pathway. Exchanging domains between TatCd and TatCy through genetic engineering produced chimeric proteins incapable of secreting the substrates of either TatC protein. Site-directed mutagenesis showed that many residues of the N-terminus are essential to the function of TatCd and TatCy. Additionally, C-terminal deletions of 5 and 8 residues produced a TatCy protein that no longer functioned (Eijlander *et al*., 2009a). This shows that the TatC proteins contain many essential regions and that alterations of the TatC proteins often lead to dysfunctional TatAC complexes.

Nearly all instances of heterologous protein expression and secretion have used the canonical Sec or Tat pathways. However, these pathways still have significant bottlenecks that impede easy heterologous protein expression. To explore the possibility of circumventing these bottlenecks, one study used the non-canonically secreted RDPE protein to secrete heterologous proteins via a hitchhiking mechanism. The proteins of interest were fused to RDPE and their presence in the extracellular medium. Two out of five heterologous bacterial proteins and both heterologous eukaryotic proteins were secreted into the extracellular medium and retained their enzymatic activity despite the fusion (Chen *et al*., 2016). This research shows that non-classical secretion pathways might be a good alternative for heterologous protein expression of proteins that cannot be secreted by Sec or Tat.

5.3 Optimization of intracellular and extracellular chaperones

Overexpression of intracellular or extracellular chaperones has been used to increase heterologous protein expression, often in combination with overexpression or deletion of other relevant proteins. These cases of combinatorial engineering have shown promising results and indicate that the improvement of multiple secretion pathway components is greater than the sum of the improvement of the individual components.

Inactivation of *hcrA* and the resulting overexpression of the DnaK and GroE chaperones resulted in a 60% increase in secreted anti-digoxin single chain antibody (SCA). Inactivation of *hrcA* was subsequently used together with inactivation of the *wprA* extracellular protease and the PrsA extracellular chaperone to increase the production of SCA by 250% (Wu *et al*., 1998). Recently, overexpression of chaperones was used to produce recombinant human fibroblast growth factor 21 (rhFGF21) for the first time. Overexpression of DnaK led to the highest increase in rhFGF21 yield, raising yields by 987% (Li *et al*., 2019). To further reduce degradation of rhFGF21 extracellular proteases were knocked out as well. rhFGF21 is a very valuable compound for treating metabolic diseases and therefore easy and plentiful production of this compound shows the enormous promise that heterologous protein production with *B. subtilis* offers (Berglund *et al*., 2009).

Overexpression of PrsA has been used to increase the secretion of many heterologous proteins. The earliest attempt resulted in a 250% increase in the secretion of AmyQ and a 200% increase in the secretion of *Bacillus licheniformis* subtilisin in response to overexpression of PrsA (Kontinen & Sarvas, 1993). More recently, *Streptococcus pyogenes* pneumolysin secretion was increased 150% by the overexpression of PrsA and *Bacillus anthracis* protective antigen secretion was increased by 250%

(Vitikainen *et al*., 2005; Williams *et al*., 2003). A more recent study reported increased heterologous protein secretion by even greater amounts by overexpressing PrsA in combination with other proteins relevant to protein secretion. Overexpression of the extracellular chaperone PrsA and the intracellular chaperone DnaK increased protein secretion of *B. licheniformis* α-amylase (AmyL) and *Geobacillus stearothermophilus* (previously *Bacillus stearothermophilus*) α-amylase (AmyS) by 700% and 1200%, respectively (Chen *et al*., 2015). Another study assessed the effect of five heterologous PrsA variants for their ability to increase the production of heterologous α-amylases from the same organisms. The organisms from which PrsA and α-amylase were tested were: *B. licheniformis*, *B. amyloliquefaciens*, *G. stearothermophilus*, *B. sonorensis* and *B. NSP9.1*. The highest increase in protein production was seen in co-expression of *B. sonorensis* PrsA and α-amylase. This combination resulted in 154% increased protein yield. In the same study, the creation of a new PrsA from *B. licheniformis* and *B. NSP9.1* PrsA increased the production of AmyL by 75% while also significantly reducing secretion stress compared to the *B. subtilis* strain expressing no heterologous PrsA (Quesada-Ganuza *et al*., 2019). Overexpression of PrsA increased the measured extracellular activity of *B. pumilus* γ-glutamyltranspeptidase by 100%. γ-glutamyltranspeptidase is required for the industrial production of L-theanine, a valuable compound for the food industry due to its taste and nutritional properties (Yang *et al*., 2019). These results show that PrsA is a worthwhile target for optimizing heterologous protein production.

5.4 Optimization of extracellular proteases

The earliest attempts to alter the protease composition of *B. subtilis* knocked out all known extracellular proteases at the time, creating WB600 (Wu *et al*., 1990). This strain lacked the NprE, AprE, Mpr, Epr, Bpf, and NprB proteases giving it only 1% protease activity compared to the original *B. subtilis* 168 strain. When new proteases were discovered these proteases were knocked out as well to create strains with even less protease activity. These WB600, WB700 and WB800 strains were used with many different heterologous proteins to achieve much higher yields than with other strains (Zhou *et al*., 2018; Ye *et al*., 2000; Phuong *et al*., 2012). Many more examples of increased heterologous protein secretion with the WB600, WB700 or WB800 strain exist but cannot all be discussed in this review. More recently, the fact that proteases do not have a universally negative influence on protein secretion led to an effort to characterize the effect of each protease on heterologous protein secretion. Large screening efforts led to second generation expression hosts that contain only the extracellular proteases that were shown to positively affect the protein being expressed (Zhao *et al*., 2019). However, the effect of each protease is not the same for every protein, thus necessitating a screening test of all proteases for each new heterologous protein to be expressed. Deletion of *htrA*, *htrB* or components of the CssRS system did not lead to increases in heterologous protein expression, further cementing the roles of HtrA and HtrB as quality control proteases that are essential for protein secretion. The secretion of AmyS and AmyL was in fact reduced by 50% when the *cssR* gene was knocked out (Vitikainen *et al*., 2005).

Most efforts to optimize *B. subtilis* as an expression host use previous efforts as a starting point. However, the 168 strain used for most optimization efforts is a domesticated strain with a tryptophan auxotrophy (Burkholder & Giles, 1947). This strain is easily transformable but does not grow as well as other less domesticated strains. In one study a more ancestral strain, ATCC 6051, was used as a starting point to engineer an optimized expression host. The *lytC* gene responsible for autolysis and the *spoIIGA* gene involved in sporulation were deleted, yielding a strain with reduced autolysis and no sporulation

but without the auxotrophies of strain 168. This ATCC 6051 strain obtained a higher optical density than the 168 strain but this did not translate into higher levels of protein secretion (Kabisch *et al*., 2013). This strain still showed promising results and another team expanded upon this work, deleting more genes that were relevant to heterologous protein production. The eight extracellular proteases listed in **Table 1** of Chapter 4.1 were deleted to reduce proteolysis of secreted heterologous proteins. Additionally, *spoIIAC* involved in sporulation and *srfC,* which is responsible for foaming of *B. subtilis* fermentation cultures, were knocked out (Clarke & Mandelstam, 1987; Coutte *et al*., 2010). This strain produced 1.48 times as much *Bacillus naganoensis* pullulanase and grew 1.73 times more than the ATCC 6051 strain it was derived from (Liu *et al*., 2018).

Concluding remarks

Remarkable progress has been made towards making *B. subtilis* an accessible host for abundant production of valuable enzymes. These efforts to optimize *B. subtilis* as an expression host have been fueled by ever-increasing knowledge about the fundamental molecular mechanisms behind protein secretion and by advancing of the tools for metabolic engineering. Most encouragingly, improving single parts of the secretion machinery increased protein expression two- or threefold but combinatorial engineering of multiple components of the secretion system raised this to over tenfold. Increases in secretion of AmyL and AmyS of 700% and 1200%, respectively, by overexpressing intra- and extracellular chaperones simultaneously shows that the effect of combinatorial engineering is greater than the sum of its parts (Chen *et al*., 2015). Extrapolating this, it can be speculated that protein yields can become much higher still when all parts of the secretion pathway described in this review are engineered together in *B. subtilis*. Moreover, abundant production of compounds of tremendous medical or industrial value, such as FGF21, shows the enormous potential of *B. subtilis* for the bio-based production of societally relevant compounds (Li *et al*., 2019).

However, the use of *B. subtilis* as an expression host remains limited to proteins that do not require post-translational modification(s). This excludes its use for the production of many proteins from eukaryotic organisms. In these cases using yeasts, fungi or other eukaryotic cells as expression hosts is the only option. Since the machinery required for post-translational modification is complex and not the same for each post-translationally modified protein, it is tremendously difficult to engineer *B. subtilis* in such a way as to express and process such proteins and it is therefore highly unlikely that this will ever be attempted.

Nonetheless, this does not diminish the value of *B. subtilis* as a heterologous protein expression host. Many proteins do not require post-translational modifications and are industrially or medically valuable and *B. subtilis* has been shown to be an excellent expression host for these proteins. Overall, the improvements to the secretion pathway components described in this review combined with innovative new approaches to genetic engineering, such as the toxin-antitoxin food-grade expression system, will make *B. subtilis* a versatile expression host for many valuable proteins (Yang *et al*., 2016).

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