Construction of an efficient secretion system for recombinant proteins in *Bacillus subtilis*

Dissertation

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Vorgelegt von

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To my parents

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Summary

All proteins being translocated through the cytoplasmic membrane of bacteria cells as well as some proteins that are inserted into the cytoplasmic membrane contain a signal sequence at their N-terminus that is recognized by and targeted to the translocation machinery. Three translocation pathways have been described, so far in *E. coli* to allow secretion of proteins: The Sec, the Tat and the SRP (Signal Recognition Particle) pathway. While the Sec and the Tat pathway act post-translationally and accept unfolded and correctly folded polypeptides, respectively, the SRP pathway acts co-translationally. For proteins secreted through the cytoplasmic membrane via the Sec pathway, the ATP-dependent motor protein SecA is required for translocation. The translocation process of some proteins following the SRP pathway has also shown to be enhanced by the presence of SecA. The Sec and the SRP pathway share the heterotrimeric protein-conducting channel translocon complex composed of the SecYEG proteins.

Based on the known characteristics of both pathways, the goal of this PhD project was to construct an efficient secretion system for recombinant proteins in *Bacillus subtilis* using an α-amylase as a reporter enzyme, which is secreted into the medium using the Sec pathway. Its gene *amyQ* was fused to an IPTG-inducible promoter. It turned out that increasing amounts of IPTG did not result in a concomitant increase of secreted α-amylase. Overproduction either formed aggregates within the cytoplasm or preproteins targeted to the translocon jammed the membrane. To release the accumulated protein within the cells two different experiments were carried out: i) a co-production and overexpression of SecA, and; ii) overexpression of an artificial *secYEG* operon. First, increased production of SecA showed significantly decrease in the total synthesis and secretion of α-amylase and did not reduce cytoplasmatic accumulation or membrane jamming. Second, the artificial operon enhanced expression of *secY*, *secE* and *secG* genes resulted in a higher amount of reporter enzyme secreted into the medium.

Furthermore, two different experiments using the transposon mutagenesis strategy were carried out in order to screen for *B. subtilis* mutants able to increase secretion of α-amylase. Transposon mutagenesis was performed with the *mariner*-based transposon to inactivate gene(s) whose product might regulate directly or indirectly the secretion of α-amylase. No mutant strain presenting a higher secretion of α-amylase on indicator plates was found. In

addition, I devised a modified transposon containing a xylose-expression cassette. To test the efficiency of the modified transposon, the promoter-less *cat* gene was used as a reporter gene and integrated into the *B. subtilis* chromosomal DNA. After transposon mutagenesis, mutants expressing the promoter-less *cat* gene were isolated. This result indicates that the modified transposon might lead to increased production of a gene in the presence of xylose and that this product might then enhance secretion of α-amylase to be detected on indicator plates.

In the third part of my thesis, a terminator-test vector was constructed which should allow the identification of strong terminators acting as 5'-stabilizing element. This vector consists of an artificial bicistronic operon containing the two reporter genes *bgaB* and *gfp* allowing the insertion of the terminators between the two genes. Insertion of a terminator should lead to a reduction of the amount of GFP. The system was verified with the known *sinIR* transcriptional terminator. It turned out that the vector with the two reporter genes already exhibited instability in *E. coli*.

Zusammenfassung

Alle Proteine, die durch die cytoplasmatische Membran transloziert werden, enthalten eine Signalsequenz an ihrem N-Terminus, welche von der Translokations-Maschinerie erkannt wird. Drei verschiedene Translokationswege wurden bislang bei *Escherichia coli* beschrieben, die die Sekretion von Proteinen erlauben: Der Sec-, der Tat- und der SRP- (Signal Recognition Particle) Weg. Während der Sec- und der Tat-Weg post-translational agieren und jeweils entfaltete und korrekt gefaltete Polypeptide akzeptieren, agiert der SRP-Weg ko-translational. Für Proteine die über den Sec-Weg sekretiert werden, spielt das ATP-abhängige SecA-Motorprotein eine essentielle Rolle beim Translokations-Prozeß. Dies trifft auf den SRP-Weg für einige Proteine zu, deren Translokation in Gegenwart von SecA gefördert wird. Der Sec- und der SRP-Weg nutzen beide das heterotrimere Translocon, welches aus den SecYEG-Proteinen besteht.

Basierend auf bekannten Charakteristika beider Wege bestand das Ziel der Doktorarbeit in der Konstruktion eines effizienten Sekretions-Systems für rekombinante Proteine in *Bacillus subtilis* unter Verwendung einer α-Amylase als Reporterenzym, welches mit Hilfe des Sec-Weges ins Medium sekretiert wird. Sein Gen *amyQ* wurde an einen IPTG-induzierbaren Promotor fusioniert. Es konnte gezeigt werden, dass erhöhte Mengen an IPTG nicht in einer gleichzeitigen Erhöhung der Menge an α-Amylase im Medium resultierte. Die Überproduktion führte zur Ausbildung von Protein-Aggregaten im Cytoplasma und einer Akkumulierung von Präproteinen an der Cytoplasma-Membran. Um die akkumulierten Proteine zu sekretieren, wurden zwei verschiedene Experimente durchgeführt: (1) gleichzeitige Überproduktion von SecA, und (2) Überexpression eines artifiziellen *secYEG*-Operons.

Eine erhöhte Produktion von SecA zeigte eine signifikante Abnahme in der Total-Synthese und Sekretion von α-Amylase und keiner Reduktion der cytoplasmatischen Protein-Aggregate und der Akkumulierung von Präprotein an der Cytoplasma-Membran. Eine induzierte erhöhte Expression der *secYEG*-Gene resultierte in einer verstärkten Sekretion des Reporterenzyms in das Medium.

Weiterhin wurde Transposon-Mutagenese mit einem *mariner*-Typ Transposon durchgeführt, in den ein Xylose-induzierbarer Promotor eingebaut worden war. Nach Transposon-Mutagenese konnten Mutanten isoliert werden, in denen ein Promotor-loses, chromosomal-lokalisiertes *cat*-Gen (Chloramphenicol-Resistenzgen) exprimiert wurde. In einem zusätzlichen Experiment sollte nachgewiesen werden, ob die Transkriptions-Terminatoren von *sinIR* und *trpA* als 3'-stabilisierendes Element fungieren unter Verwendung des *bgaB*-Reportergens.

Im dritten Teil meiner Dissertation wurde ein Terminator-Testvektor konstruiert, der die Identifizierung starker Terminatoren erlauben sollte, die als 5'-stabilisierende Elemente fungieren. Dieser Vektor besteht aus einem artifiziellen bicistronischen Operon mit den beiden Reportergenen *bgaB* und *gfp*, und der Terminator kann zwischen beide Gene eingebaut werden. Der Einbau eines Terminators sollte zur einer Reduktion der Menge an GFP führen. Dieses System wurde mit dem bekannten *sinIR* Transkriptions-Terminator getestet. Es zeigte sich, dass bereits der Vektor mit den beiden Reportergenen in *E. coli* instabil war.

1 Introduction

1.1 Protein traffic: The key role of signal peptides

Almost all bacterial proteins are synthesized by ribosomes within the cytosol and 25 to 30% of these proteins function within the cell envelope or outside of the cell (Driessen and Nouwen, 2008). Therefore, these secretory proteins, so-called preproteins, have to be transported to the cell membrane or throughout the cell wall to fulfil their function. The cell envelope must allow and control the secretion of proteins as well as act as a protective barrier to maintain the transport of cytoplasmic molecules from and to the extracellular compartment. In the Gram-positive bacterium *Bacillus subtilis*, the cell envelope is mainly composed by many layers of peptidoglycan that surround the cytoplasmic membrane. This characteristic appears to simplify the protein translocation process in *B. subtilis* since its cell structure is less complex than the structure present in eukaryotic cells where a complex network of membranes separates different organelles (Kelly, 1985). It is also simpler than that of the Gram-negative bacteria, such as *Escherichia coli*, where the cell envelope is divided into four compartments: cytosol, inner membrane, periplasm and outer membrane (Hobot *et al*., 1982).

The translocation process of proteins became elucidated in the 70's, when C. Milstein and colleagues discovered that preproteins are synthesized as cytosolic precursor proteins with an amino-terminal extension called signal peptide (SP) (Milstein *et al.*, 1972). This segment plays a key role in the secretion process since the first step involves the recognition and targeting of the SP by the translocation machinery. Subsequently, the preproteins can achieve their destination in the cytoplasmic membrane, cell wall, or growth medium (von Heijne, 1998; Tjalsma *et al.*, 2000; Simonen and Palva, 1993). The SPs share common features conserved in different organisms. It consists of three distinct regions: (i) a positively-charged amino-terminus called N-region, (ii) a central, hydrophobic core where minimum hydrophobicity is required for function, called H-

region, and (iii) a carboxyl terminal-end containing a specific signal peptide cleavage site that is recognized by signal peptidases (SPase), called C-region. Each region has been shown to be essential for the functioning of the SP (von Heine and Abrahmsen, 1989; Gierasch, 1989; Hikita and Mizushima, 1992; Schatz and Dobberstein, 1996; Zanen *et al.*, 2005; Brockmeier *et al.*, 2006a; Gouridis *et al.*, 2009). It is typically composed of 15 to 25 amino acid residues and in the *Bacillus* species; they are usually five to seven amino acids longer than those of *E. coli*. The extension takes place in all three regions (N-, H-, and C-) and in addition, the *Bacillus* N-region usually contains a higher number of positively charged lysine and arginine residues (Driessen and Nouwen, 2008).

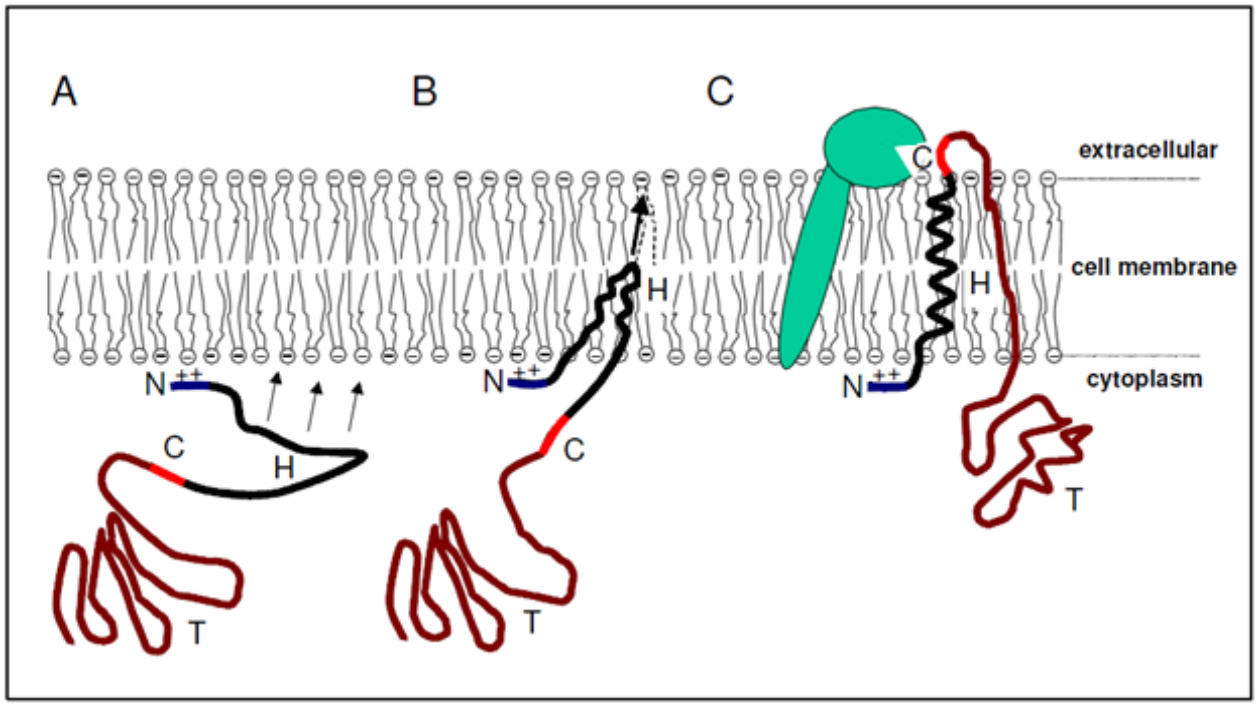

Figure 1: Simplified model for the interaction of a signal peptide with a cell membrane
A SP is composed of a positively charged N-region (N- highlighted in blue), a central, hydrophobic region (H- highlighted in black) and the cleavage site (C- highlighted in red). The SP is fused to the targeted protein (T) forming the preprotein. The high tendency of the SP to insert into the membrane is indicated by black arrows. The SPase is coloured in green. For clarity, cytoplasmic targeting factors (e.g. SRP, SecA, SecB) or other components essential for protein secretion are not considered in this model (Brockmeier, 2006).

The SP is inserted into the cytoplasmic membrane in a three-step mechanism (Fig. 1). In the first step the positively charged N-region, containing at least one arginine or lysine residue, interacts with the negatively charged phospholipids of the membrane (Fig. 1 A) (Akita *et al.*, 1990).

While the N-region stays at the cytoplasmic side of the membrane, the H-domain continuously inserts into the membrane adopting an α-helical conformation due to the presence of helix-breaking glycine or proline residues (Fig. 1 B). Due to the process of unlooping of the hairpin-like structure, the complete SP is inserted pulling the N-terminal part of the preprotein throughout the cytoplasmic membrane, therefore exposing the C-region at the trans-side of the membrane where a specific SPase recognizes and cleaves the SP from the mature part of the exported protein during translocation or shortly after (Fig. 1 C) (Paetzel *et al.*, 2002; Van Roosmalen *et al.*, 2004). Finally, the mature part of the protein is released from the membrane and can fold into its native conformation.

The last step concerning processing of the SP by a SPase is a mandatory reaction to release the mature secretory protein. This step involves the type I SPase. Five genes for type I SPase have been identified in separated regions of the chromosome of *B. subtilis*, denoted *sipS, sipT, sipU, sipV and sipW* (van Dijl *et al.*, 1992; Tjalsma *et al.*, 1997). H. Tjalsma and colleagues demonstrated that SipS and SipT are the most important SPases of *B. subtilis*, whereas SipU, SipV, and SipW appear to have a minor role in processing of the secretory proteins (Tjalsma *et al.*, 1997). The type I SPases of eubacteria, mitochondria, and chloroplasts differ considerably in their essential amino acid residues from their homologs in Archaea and in the endoplasmatic reticulum membrane (ER), indicating that these enzymes belong to two distinct subfamilies of SPases (Dalbey *et al.*, 1997;Van Roosmalen *et al.*, 2004). In this regard, *B. subtilis* has been shown to be the first organism known to contain type I SPases of both subfamilies present in one membrane. In contrast, *E. coli* contains only one type I SPase and most eukaryotic cells contain two type I SPases, however in different membranes (Yamane *et al.*, 2004; Tjalsma *et al.*, 1998).

Small variations in the SP structure and the SPase involved in the processing of the SP are crucial characteristics to lead the transport of proteins to different destinations and/or export via different pathways. Regarding these features, *Bacillus* SPs are classified into four major classes that are distinguished by their export pathway and their SPase cleavage sites: (i) secretory (Sec-type) signal peptides, present in preproteins, targeted to the secretion pathway (Sec pathway) or the SRP (Signal Recognition Particle) pathway, cleaved by type I SPase, (ii) Tat-dependent

signal peptides targeted to the TAT (Twin Arginine Translocation) pathway, (iii) lipoprotein signal peptides cleaved by the type II SPase, and (iv) propeptides, located in the primary translation product between its SP and the mature protein defined as a folding factor, responsible for accelerating the folding process and stabilizing the protein (Fig. 2) (Harwood and Cranenburgh, 2008).

This study will only focus on the Sec-type SPs which are recognized and targeted either by the general secretion pathway (Sec pathway) or the SRP (Signal Recognition Particle) pathway. Both pathways are described in detail in the next chapters.

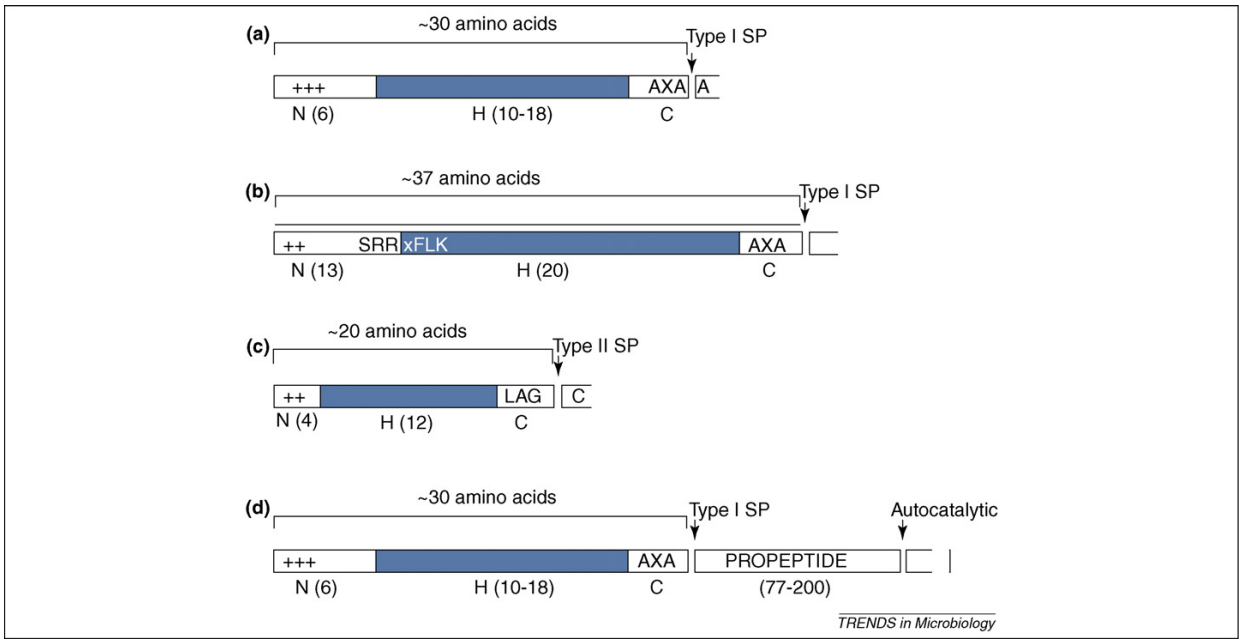

Figure 2: General features of the SP of *Bacillus* secretory proteins
The predicted SPs were divided into four distinct classes on the basis of their SPase cleavage site and the export pathways. Amino acids are shown in the one-letter code. "X" is defined as any amino acid. The N- and the C-regions are distinguished by white colored boxes flanking the H – region colored in blue. Cleavage sites are indicated by arrows. The N-terminal part of the mature protein is indicated as a white open box. (**a**) Sec-type SPs are targeted across the cytoplasmic membrane by the general secretion pathway (Sec pathway). The cleavage site is recognized by one of the five type I SPases at the AXA cleavage site. (**b**) Some secretory proteins were identified containing a SP with the consensus motif SRRxFLK and cleaved by a type I SP indicating the potential to be secreted by the Tat pathway. Tat signal peptides are generally longer and less hydrophobic than their counterparts in Sec-type SPs. (**c**) Lipoprotein signal peptides are cleaved by the type II SPase (LspA) and share discrete characteristics that include shorter N- and H- regions and a cleavage sites named lipobox with the consensus motif L-X-X that is distinct from that of type I signal peptides. (**d**) The signal peptide and propeptide (prepropeptide) at the N-terminal end of a secretory protein requiring the propeptide for folding on the trans-side of the cytoplasmic membrane (Harwood and Cranenburgh, 2008).

1.2 Secretion of proteins: The pathways

Initially, the secretory and the membrane proteins are synthesized by the ribosomes as extended polypeptides that have not yet folded into their final conformation. Since unfolded proteins are unstable in the cytosol, these polypeptide chains need to be immediately stabilized by cytosolic chaperones in order to prevent folding and/or aggregation prior to their translocation (Ben Zvi and Goloubinoff, 2001). The molecular chaperones are responsible for keeping the preproteins in an export-competent state. Now, they are prone to be targeted to the membrane-embedded translocon, a pore-conducting channel named SecYEG that allows translocation of secretory proteins across the cytoplasmic membrane and also contains a lateral gate involved in the insertion of the membrane proteins (Driessen and Nouwen, 2008; Dalbey *et al.*, 2011).

To reach their destination the precursor protein can follow two routes: a post-translational translocation, where the synthesis of the precursor protein is complete before its translocation; or a co-translational translocation, a mode in which the synthesis of the precursor protein is coupled to the translocation machinery, i.e. the protein is targeted as ribosome-bound nascent chains (RNCs) at the same time that its translocation takes place.

1.2.1 The post-translational translocation mechanism: The Sec pathway and the role of SecB and SecA in *E. coli*

The post-translational translocation mechanism in *E. coli*, described in the literature as the Sec-pathway, targets most secretory proteins in this organism. The molecular chaperone that ensures the translocation-competent state of most preproteins is called SecB (Kumamoto and Beckwith, 1983; Kumamoto and Beckwith, 1985; Fekkes and Driessen, 1999). It is characterized as a homotetrameric protein organized as a dimer of dimers (Xu *et al.*, 2000). The mechanism by which SecB differentiates between secretory and non-secretory proteins remains poorly understood. Although SecB-binding sequences have been identified, these motifs appear in the same frequency in secretory as well as in cytoplasmic proteins (Knoblauch *et al.*, 1999).

However, it is known that SecB only interacts with unfolded nascent secretory proteins and appears to associate with mature regions that are normally buried in the folded structure (Randall *et al.*, 1998). In this regard, the role of the signal peptide that characterizes a secretory protein is thought to be involved in modulation of the folding process in order to expose fragments from the polypeptide chain that are recognized by SecB (Randall *et al.*, 1990).

A key feature that distinguishes SecB from other chaperones is its ability to interact with high affinity with the SecA protein, the central component of the Sec pathway, which functions as an ATP-dependent motor protein (Hartl *et al.*, 1990; Randall and Henzl, 2010). The SecA-SecB complex occurs with high affinity at the membrane surface by an interaction between the negatively charged N-terminus present on both sides of SecB and the C-terminal zinc-containing domain of SecA (Randall and Henzl, 2010). This model of the SecB-SecA complex suggests that this interaction is responsible for changing the conformation of the polypeptide binding site in SecB, therefore enabling transfer of the preprotein from SecB to SecA (Zhou and Xu, 2003; Zhou and Xu, 2005). Once the preprotein is attached to SecA, its ATPase activity is required to initiate translocation of the precursor. At this very initial stage of the translocation SecB is released from the complex due to a large conformational change of SecA upon binding with ATP (Fekkes *et al.*, 1997). The SecA protein also interacts with other components involved in protein translocation such as acidic phospholipids present in the cytoplasmic membrane, the SecYEG translocon, ribosomes, and SP as well as mature parts of the preprotein (Lill *et al.*, 1990; Miller *et al.*, 2002; Karamyshev and Johnson, 2005; Papanikou *et al.*, 2005; Zimmer and Rapoport, 2009).

Briefly, SecA is divided into several subdomains: two nucleotide binding domains (NBD1 and NBD2), where the conversion of chemical energy into movement is performed; a preprotein crosslinking domain (PPXD), where the interaction SecA-preprotein takes place; an α-helical scaffold domain (HSD), which contacts all other domains of SecA, therefore playing an important role in the catalytic cycle of SecA; and a C-terminal translocation domain (HWD and CTL) (Driessen and Nouwen, 2008; Papanikou *et al.*, 2005; Yuan *et al.*, 2010).

SecA localizes both to the cytosol and the cytoplasmic membrane and exists in a dynamic equilibrium between a monomeric and a dimeric form, being mainly dimeric in the cytosol or, when purified, and displayed in an antiparallel orientation (Ding *et al.*, 2003; Sardis and Economou, 2010). Remarkably, crystal structures of both monomer and dimer SecA have been observed to be bound to SecYEG using different biochemical approaches. Therefore, the oligomeric state of SecA during the translocation process has been a topic of significant controversy. Taken together, the pool of data regarding the role of different oligomeric forms of SecA suggests the current hypothesis. The dimeric form assumes the function of a cytosolic chaperone that guides preprotein to the SecYEG translocon or the dimeric form is irrelevant only if the SecA monomerizes upon binding to SecYEG and subsequently catalyzes secretion as a monomer (Sardis and Economou, 2010; du Plessis *et al.*, 2010).

1.2.2 **The protein-conducting channel: SecYEG**

The process of protein translocation across the cytoplasmatic membrane is performed throughout an evolutionary conserved heterotrimeric protein complex designated as SecYEG in bacteria and Sec61αβγ in eukaryotes (Mandon *et al.*, 2009). This protein-conducting channel is designated as translocon. In bacteria, the translocon consists of three proteins named SecY (homologous to Sec61α), SecE (homologous to Sec61β and Sec61γ) and SecG. The first high-resolution structure reported was that of the archaea SecYEβ complex of *Methanocaldococcus jannaschii* (Van den *et al.*, 2004). One year later, the crystal structure of the *E. coli* SecYEG revealed that the two complexes differ only slightly in conformation (Bostina *et al.*, 2005), providing many new insights into the structure of the translocon in this organism.

E. coli SecY is the largest component of the translocon and is essential for viability and translocation (du Plessis *et al.*, 2010). Its structure spans the membrane ten times in an α-helical conformation. The N- and C- domains comprise the 1-5 transmembrane segments (TMS) and 6-10 TMS, respectively (Driessen and Nouwen, 2008). SecE, akin to SecY, is essential for viability and translocation. It is a small integral membrane protein with three TMS. Interestingly, only the third segment is required for function and this very one corresponds to the unique TMS Sec61γ

present in eukaryotes and to the single TM SecE present in other bacteria (Murphy and Beckwith, 1994). SecG is localized at the periphery of the complex (Breyton *et al.*, 2002). It is a small molecule containing one TMS and, differently from SecY and SecE, it is not essential for viability and translocation. Although it makes only limited contact with SecY, there is no evidence of interaction between SecG and SecE (Homma *et al.*, 1997).

The model for the structure of the translocon proposes that the protein-conducting channel is shaped like an inverted funnel where SecA, the ribosome, and the polypeptide chain likely interact with the channel. This structure is due to the interaction between SecY and SecE. SecY is embraced by SecE in both the N- and C- domains by a loop between TMS5 and TMS6 and this connection forms a central pore ring in the structure whereby the preprotein is inserted into the translocon. This pore ring constricts the channel due to the presence of hydrophobic amino acid residues which have their hydrophobic side chains towards the center of the channel maintaining the permeability of the membrane during translocation (Ding *et al.*, 2003). Moreover, it has been shown that the assembly or the stability of the bacterial SecYEG is influenced by the preprotein that needs to be translocated (Boy and Koch, 2009).

From the periplasmic side of the cytoplasmatic membrane a "plug" is formed into the funnel-like cavity. This structure is displaced upon binding of the preprotein leading to the opening of the pore ring, accompanied by an overall expansion of SecY, consequently allowing the insertion and translocation of the preprotein to occur. Besides guiding the unfolded protein towards the extracellular compartment it has also been proposed that, upon the binding of SecA, this "plug" displacement plays a very important role in the lateral opening of the translocon mechanism by which the majority of membrane proteins are inserted (Driessen and Nouwen, 2008; Ding *et al.*, 2003; Zimmer *et al.*, 2008; Egea and Stroud, 2010).

Along with SecA, the oligomeric state of SecYEG is under a considerable controversy. The translocon can be found in a dynamic equilibrium between monomers, dimers, and even higher-order oligomers. Association of monomeric SecA-SecYEG was suggested to be sufficient for protein translocation (Zimmer *et al.*, 2008). However, other studies have reported that a

monomeric SecA is bound only to the SecYEG dimer, suggesting that the binding of SecA shifts the equilibrium of the monomeric SecYEG towards to the oligomeric state (Manting *et al.*, 2000; Duong, 2003; Scheuring *et al.*, 2005).

The Sec components have been described in both *E. coli* and *B. subtilis* to be localized into a spiral-like structure along the cell (Campo *et al.*, 2004; Shiomi *et al.*, 2006). In *B. subtilis* most of the translocons are organized in specific clusters along these structures particularly during the exponential growth phase. It also appears to be independent to the helicoidal structures formed by the actin-like cytoskeleton. Interestingly, SecA presents a dynamic localization depending on both membrane-phospholipids composition and the level of synthesis of exported membrane proteins (Campo *et al.*, 2004).

In prokaryotes, the SecYEG channel can associate with another heterotrimeric-membrane complex consisting of the SecD, SecF, and YajC proteins (Pogliano and Beckwith, 1994). All three proteins were identified in *B. subtilis* differing in two aspects from their equivalents in *E. coli*. *B. subtilis* SecD and SecF are present in a single polypeptide chain denoted SecDF that is required to maintain a high capacity of protein secretion. However, SecDF is not required for the release of a mature secretory protein from the membrane indicating its involvement in earlier translocation steps. The YajC homolog in *B. subtilis* is encoded by the *yrbF* gene and is located in a locus separated from *secDF* (Bolhuis *et al.*, 1998). Further studies are required to enlighten the unknown mechanism by which SecDF influences protein secretion in *B. subtilis*.

1.2.3 **The Sec pathway: The mechanism of post-translational translocation**

In the post-translational translocation process, the synthesis and translocation of the preproteins are uncoupled events. After the preprotein has been synthesized on the ribosomes, it can be bound to SecB which targets the preprotein to the translocon in a translocation-competent state. The energy necessary for protein insertion and translocation in the post-translational mode is provided by ATP-hydrolysis at SecA and by the Proton Motive Force (PMF). While ATP is essential for the initiation of translocation as well as during the whole process, the PMF appears

to affect only the orientation of the preprotein once it is inserted into the pore ring of the translocon by changing the conformation of SecY (Driessen and Nouwen, 2008; Driessen, 1992; Duong, 2003).

Fig. 3 shows in detail how the entire mechanism of protein translocation via the Sec pathway is performed in bacterial cells.

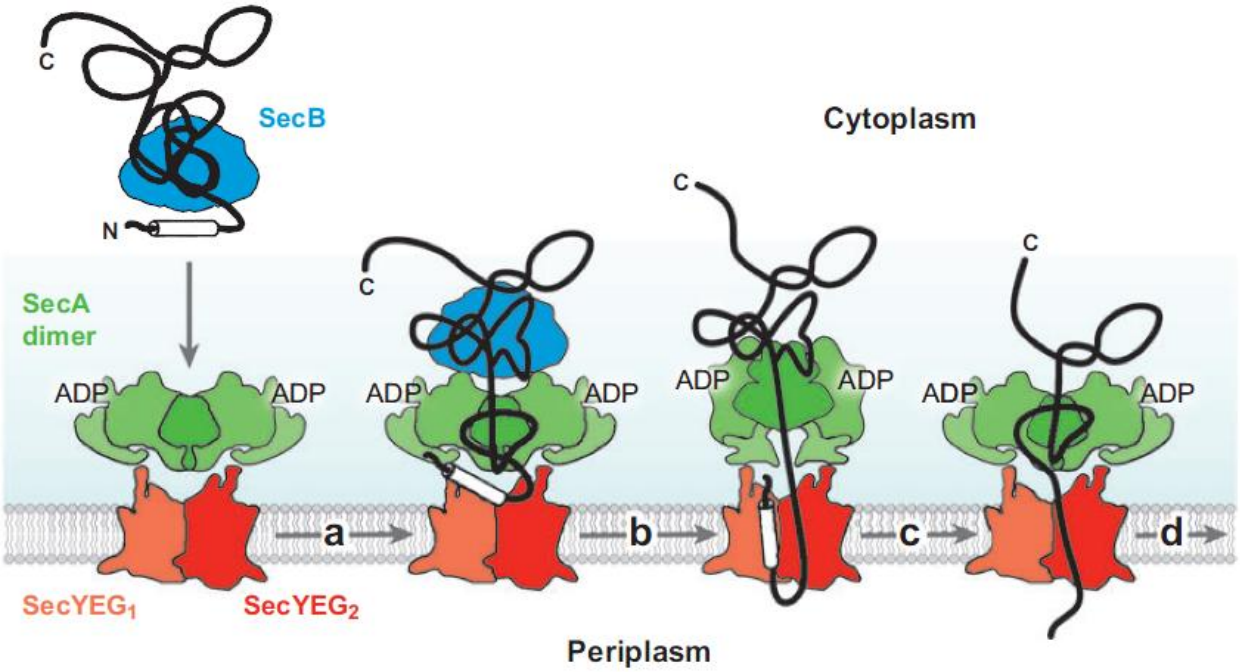

Figure 3: Model of bacterial post-translational translocation (or Sec-mediated protein translocation).
The protein is represented by the black line with the white region showing the SP. During the first step, the SP is recognized and bound by the SecB chaperone (coloured in blue). The SecA dimer (colored in green) is bound to the dimer of SecYEG (coloured in red) in an open conformation, creating a large central cavity in between SecA and SecYEG. (**a**) In this state, it accepts the preprotein from SecB, which has prevented stable folding or aggregation of the preprotein. Due to Brownian motion, the polypeptide passes through the central opening in SecA into the cavity where the SP is bound and the cavity fills up with protein. (**b**) Conformational changes (grey arrow) due the binding of ATP result in the release of SecB and closure of the SecA central opening concomitantly with an opening of the SecYEG channel. Consequently, there is a reduction of the cavity volume and the release of a segment of the preprotein is performed. (**c**) ATP-hydrolysis reverses the SecA conformational change, which results in the reopening of the central SecA channel and closure of the SecYEG pore, allowing a new stretch of the secretory protein to enter the cavity. (**d**) This cycle of nucleotide-dependent, alternating opening and closing of the central opening in the SecA and the pore in SecYEG is repeated until translocation of the polypeptide is completed. It is believed that multiple rounds of ATP-binding and hydrolysis lead to a stepwise translocation of the preprotein, whereby each step results in the translocation of approximately 5 kDa (Driessen and Nouwen, 2008; Rapoport, 2007; van der Wolk *et al.*, 1997).

1.2.4 **The co-translational translocation mechanism: The role of SRP and its receptor**

The co-translational translocation mediates the transport of secretory and membrane proteins to the plasma membrane in prokaryotes, and it is also the major pathway to transport membrane proteins to the endoplasmic reticulum in eukaryotes (Luirink and Sinning, 2004; Grudnik *et al.*, 2009; Egea *et al.*, 2005). Studies in *E. coli* also showed that a set of inner membrane proteins is assembled by the co-translational translocation mode (Beck *et al.*, 2000; De Gier *et al.*, 1996; De Gier *et al.*, 1998; Koch *et al.*, 1999; Ulbrandt *et al.*, 1997; Valent *et al.*, 1998).

This mode also involves a precursor protein in a translocation-competent state, i.e., an unfolded state compatible with translocation. No *secB* gene has been identified in *B. subtilis*. But an unrelated gene designated *csaA* has been described that might act as a holder chaperone to prevent folding of polypeptide chains to be translocated (Müller *et al.*, 2000; Shapova and Paetzel, 2001). Although the evidence for its role in secretion needs to be confirmed experimentally, CsaA has been shown to interact with SecA, to bind peptides and it is upregulated under secretion stress (Linde *et al.*, 2003; Müller *et al.* 1992; Vitikainen et al, 2005). Moreover, although *B. subtilis* contains molecular chaperones such as GroE and DnaK, a specific role of these chaperones in secretion of endogenous proteins in this organism has remained vague (Mogk *et al.*, 1997; Schmidt *et al.*, 1992; Wetzstein *et al.*, 1992; Wiegert and Schumann, 2003; Wiegert *et al.*, 2004; Wu *et al.*, 1998). Given that in *B. subtilis* cells there is no homolog to *E. coli* SecB, the secretion and insertion of proteins in the cytoplasmatic membrane is likely to happen in a co-translational manner (Tjalsma *et al.*, 2000; van Wely *et al.*, 2001). To date, the best candidate to be the functional counterpart of SecB in *B. subtilis* is the SRP.

The SRP is conserved in all three kingdoms of life (Fig. 4). It was first described in eukaryotic cells consisting of one 7S RNA molecule (SRP-RNA) containing approximately 300 nucleotides and six proteins named according to their molecular weight: SRP72, SRP68, SRP54, SRP19, SRP14 and SRP9 (Walter and Johnson, 1994). The SRP-RNA is divided into two domains that define structurally and functionally distinct parts: SRP14 and SRP9 bind to the end of the SRP-RNA to form the Alu domain with a function in elongation arrest during SP targeting. The

central region of the SRP-RNA together with SRP72, SRP68, SRP19 and SRP54 constitute the S domain in which SRP54 carries the binding site for the SP and the interaction site with the SRP receptor (SR) embedded in the cytoplasmic membrane (Doudna and Batey, 2004; Walter and Johnson, 1994) (Fig. 4).

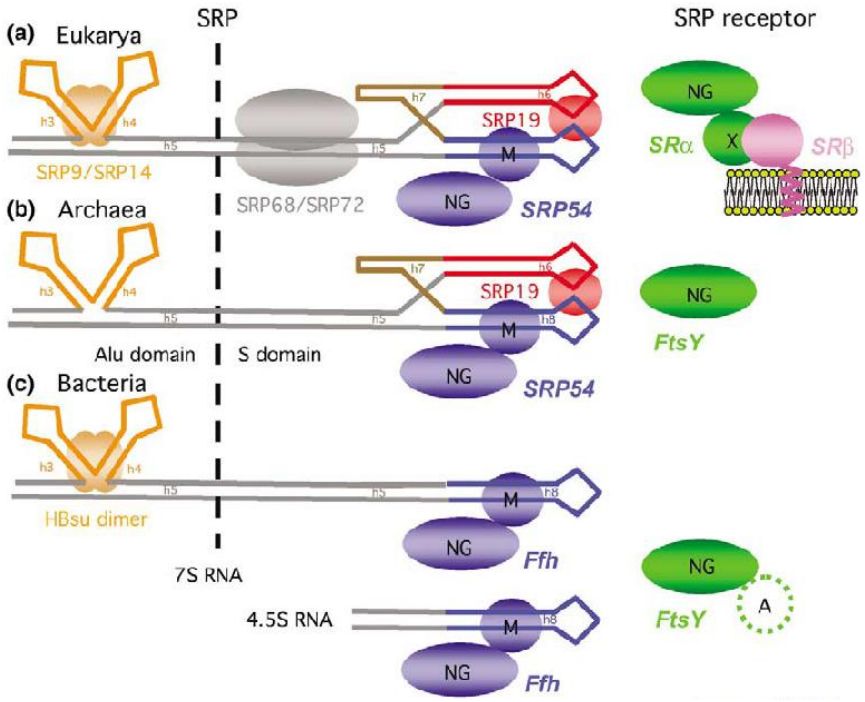

Figure 4: The SRP and its receptor (SR) through evolution
Schematic representations of the architecture of the SRPs and SRs from (**a**) Eukarya, (**b**) Archaea and (**c**) Bacteria. The GTPases (Ffh/SRP54 and FtsY/SRα) are indicated in bold with the N- and G- domains. The RNA helices present in Eukaryotes and Archaeal SRP-RNA (~300 nucleotides), and in Bacteria 4.5S RNA (~115 nucleotides) are labeled h2 through h8. In SRP54/Ffh, the M-domain is responsible for SP recognition. The N- and G- domains of SRP and SR are closely related and responsible for the GTPase activity. Some bacterial FtsY proteins contain an extra N-terminal A-domain not present in *B. subtilis*. The eukaryotic SR is composed of two subunits: The regulatory subunit SRβ (containing an N-terminal transmembrane anchor) and SRα. Some gram-positive bacteria, such as *B. subtilis*, retain a long SRP-RNA with an Alu-like domain to which a dimeric protein (HBsu) is bound, as SRP9/SRP14 in eukaryotic SRP (Egea *et al*., 2005)

SRP54 is divided into three domains termed N-, G- and M-domains. The M-domain is rich in methionine residues which are believed to form a groove to accommodate the SP of the preprotein. It is additionally responsible for binding to the SRP-RNA. The G-domain has a

GTPase activity that increases the efficiency of SP-binding and is involved in both the recognition and binding to the SR. The N-domain is structurally and functionally coupled with the G-domain (Clemons, Jr. *et al.*, 1999; Luirink and Dobberstein, 1994).

The *E. coli* SRP is composed of a small 4.5S RNA and a single protein homologous to SRP54, termed Ffh (Fifty four homolog) (Phillips and Silhavy, 1992; Miller *et al.*, 1994). The *B. subtilis* SRP retains Ffh and a small cytoplasmic RNA (scRNA) of 271 nucleotides with an Alu-like domain to which a dimeric protein termed HBsu is bound, akin to SRP9/SRP14 in the eukaryotic SRP (Struck *et al.*, 1989;Nakamura *et al.*, 1992;Honda *et al.*, 1993). The HBsu, Ffh and scRNA make a stable complex where the scRNA functions as a backbone for complex formation and this complex can be located in both membrane and cytoplasm compartments (Nakamura *et al.*, 1994;Nakamura *et al.*, 1999). It has been shown that depletion of Ffh led to defective production of extracellular enzymes as well as morphological changes (Honda *et al.*, 1993). Furthermore, changes in the structure of the scRNA affect the viability of cells, cell growth and production of spore-related proteins (Nishiguchi *et al.*, 1994). The SRs are also phylogenetically conserved (Fig. 4).

In eukaryotes, the SR consists of two subunits: the peripheral membrane protein SRα and the integral membrane protein SRβ. Bacterial SRs are single subunit proteins named FtsY, a homolog of SRα in Eukaryotes. The SRα as well as the FtsY are multidomain proteins that share two conserved N- and G-domains like those present in SRP54 and Ffh. *E. coli* FtsY and mammalian SRα contain an additional acidic domain at their N-terminus, called A-domain, which is proposed to be involved in association with the membrane. This domain is not present in *B. subtilis* FtsY (Yuan *et al.*, 2010; Luirink and Sinning, 2004; Egea *et al.*, 2005).

The GTPase activity of both SRP and SR GTPase regulates protein translocation. Their interaction responds to various external regulators in the ribosome, the SecYEG channel, the SP and the complex formation between SRP and SR (Luirink and Sinning, 2004; Grudnik *et al.*, 2009; Bradshaw and Walter, 2007). In order to ensure the activity of the complex SRP-SR their GTPases have to be synchronized in a GTP-bound state forming a heterodimeric complex where

the GTPases are then mutually stimulated, acting as GTPase activating proteins for each other. In addition, the SRP-type GTPase contains an insertion box domain, which moves into close proximity to the substrate and contributes to additional interactions at the heterodimer interface (Shan and Walter, 2005). Following release of the preprotein from SRP into the translocon machinery, hydrolysis of SRP- and SR-bound GTP molecules causes dissociation of the SRP-SR complex.

The *B. subtilis* SecA also plays an important role in the insertion of proteins by the co-translational translocation mode (Takamatsu *et al.*, 1992). The *B. subtilis* Ffh as well as *E. coli* SecB enhance the binding of SecA to the preprotein indicating that they interact cooperatively during the translocation process (Bunai *et al.*, 1999). Moreover, the interaction between SecA and the SRP is required to insert lipoproteins and most membrane proteins into the cytoplasmatic membrane in *B. subtilis* and in *E. coli* (Hirose *et al.*, 2000; Qi and Bernstein, 1999; Yamane *et al.*, 2004; Valent *et al.*, 1998).

Fig. 5 shows in detail how the entire mechanism of protein translocation via the SRP pathway is performed. In general, the SP recognized by *B. subtilis* SRP is known to have a clear preference for the most hydrophobic signals (Zanen *et al.*, 2005). Interestingly, a proteomic study showed that the extracellular accumulation of individual proteins was found to be affected, to different extents, by depletion of Ffh or FtsY. In addition, no clear correlation between reduced extracellular amounts of different proteins and the hydrophobicity of their SPs was observed suggesting that the SP hydrophobicity is not the only factor that determines the Ffh dependence of a secretory protein (Zanen *et al.*, 2006). These results suggest that others, so far unidentified determinants are also important for SRP-dependence in the secretion of proteins in *B. subtilis*. Besides, U. Brokmeier and coworkers demonstrated that the best SP for the secretion of a heterologous protein is not automatically adequate for the secretion of another protein and that there is no correlation to the secretion efficiency and length, charge, or hydrophobicity of the SP (Brockmeier *et al.*, 2006a).

On the other hand, several studies in *E. coli* have shown that the SRP-dependent SPs are significantly more hydrophobic and also that by increasing the hydrophobicity of the SP, membrane proteins can reroute into different pathways (Huber *et al.*, 2005; Schierle *et al.*, 2003; Lee and Bernstein, 2001; Zanen *et al.*, 2006; Valent *et al.*, 1998; De Gier *et al.*, 1996).

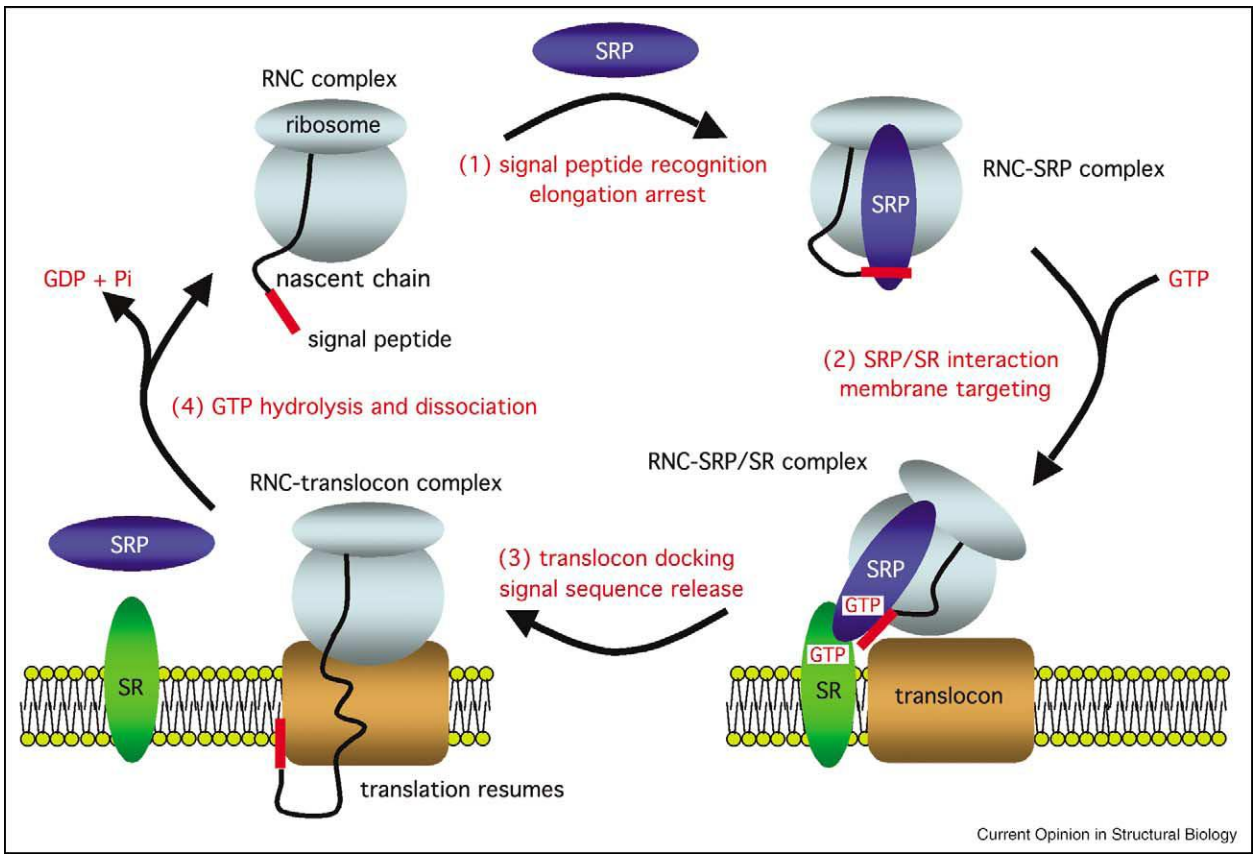

Figure 5: Schematic representation of the SRP protein secretion pathway
The protein is represented by the black line with the red region showing the SP. In the first step (**1**), the SP is recognized and bound by SRP as it emerges from the ribosome. (**2**) This complex subsequently interacts with the membrane-bound receptor FtsY. (**3**) GTPase activates both FtsY and Ffh allowing release of the nascent chain to the SecYEG translocon. (**4**) GTP-hydrolysis causes dissociation of the SRP-SR complex. SecA is not considered in this model (Egea *et al.*, 2005).

1.2.5 **The YidC pathway**

Operating in parallel with the SecYEG is the evolutionarily conserved YidC pathway for the insertion of proteins into the cytoplasmic membrane of bacteria, mitochondria and chloroplasts of eukaryotes (104). YidC is an essential protein in *E. coli* and functions as a membrane insertase

playing an important role in the insertions of a subset of membrane protein. Recently, it has also been shown to greatly contribute to gene expression in this organism (Wang *et al.*, 2010). In addition to working in cooperation with SecYEG, YidC can also insert proteins on its own or insert preproteins that require SRP for membrane targeting. Nevertheless, how these proteins are correctly targeted to YidC instead to SecYEG, or even directly recognized by YidC, is an open question. And another important unanswered question is how the SRP can discriminate between preproteins to be targeted to SecYEG or to YidC (Serek *et al.*, 2004;Kol *et al.*, 2008).

In *B. subtilis*, two paralogs of YidC are present: SpoIIIJ and YqjG. The presence of either SpoIIIJ or YqjG is required for cell viability. Together these *B. subtilis* homologues have been reported to be involved in membrane-protein biogenesis rather than in protein secretion. However, it has also been shown that secretory proteins produce a reduced stability in SpoIIIJ/YqjG-depleted *B. subtilis* cells (Saller *et al.*, 2009).

1.3 The organism: *B. subtilis*

The Gram-positive soil bacterium *B. subtilis* has been widely used in the industry for decades. Tremendous protein-export capacity with yields up to 25 g/l of extracellular enzymes is one of the various advantages of using this bacterium as a cell factory in pharmaceutical, food, biotechnology, and agricultural fields (Freudl, 1992; Schallmey *et al.*, 2004; Westers *et al.*, 2004; Ferreira *et al.*, 2005; Zweers *et al.*, 2008; Nijland and Kuipers, 2008). Table 1 gives an overview of homologous and heterologous protein production in *B. subtilis* strains with respect to industrial applications.

Other advantages that make *B. subtilis* one of the best understood and extensively used in both applied and fundamental scientific research over the last years include: (i) It is a non-pathogenic bacterium and has been awarded the GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration like most of its closest relatives. (ii) It develops genetic

competence for DNA binding and uptake. (iii) It shows easy culturing conditions and excellent fermentation capacity. (iv) It has its entire genome sequenced and its essential genes are all identified (Kunst *et al.*, 1997; Kobayashi *et al.*, 2003). Another important feature is that *B. subtilis* lacks an outer membrane present in the Gram-negative bacteria, thus allowing a direct protein-export into the extracellular medium. This characteristic may avoid intracellular accumulation and provide better folding conditions, therefore simplify further purification steps (Westers *et al.*, 2004).

Although secretion of several homologous and heterologous proteins has been very successful in *B. subtilis* (Table 1), a very high yield of heterologous proteins still presents some limits for this organism in its industrial applications.

Table 1: Protein production of commercial interest in *B. subtilis*

Product	Application	Origin	Reference
Alkaline protease	detergents	*B. subtilis* *B. licheniformis*	(Rao *et al.*, 1998)
β-Glucanase	glucanase modification	*B. subtilis*	(Borriss *et al.*, 1989)
Xylanase	food processing	*B. subtilis*	(Kuhad *et al.*, 1997)
Growth hormone	medicine	human	(Hartley, 1988)
Interleukin-1beta	medicine	human	(Schallmey *et al.*, 2004)
Proinsulin	medicine	human	(Schallmey *et al.*, 2004)
Penicillin G acylase	medicine	*B. megaterium*	(Yang *et al.*, 2001)
Purine nucleotides	medicine, flavor enhancer	*B. subtilis*	(Schallmey *et al.*, 2004)
Streptavidin	biotin-binding protein	Streptomyces species	(Wu and Wong, 2002)
Antigen displaying spore	oral vaccination	tetanus toxin fragment C	(Duc *et al*., 2003)

(Modified from Brockmeier, 2006)

The production of "foreign" proteins might be a serious problem due many different reasons, e.g., an expression and secretion system developed for one protein does not ensure it can be applied for a different protein. Furthermore, every step in protein production and secretion can be a bottleneck that limits the yield of the protein (Nijland and Kuipers, 2008). During recent years many strategies have been studied in detail to overcome almost every shortcoming of the *B. subtilis* system. Some of most important bottlenecks, and possible solutions to overcome them, are discussed below.

(I) For secretion of heterologous proteins into the medium, the SP plays an important role in the efficient translocation across the membrane, labeling and directing them very efficiently to the translocon machinery at the cytoplasmatic membrane (Fig. 1). Several studies demonstrated that an increased expression of a SP can depend on different modifications such as length, hydrophobicity level and structural changes, to enhance the capacity of the secretion system of heterologous proteins in *B. subtilis* (Palva *et al.*, 1982; Meens *et al.*, 1993; Zanen *et al.*, 2005; Brockmeier *et al.*, 2006a). Moreover, is has been reported that not only the SP but also the N-terminal residues of the mature protein can be engineered to positively influence processing and secretion efficiency (Simonen and Palva, 1993).

(II) To date, in most cases for the production of heterologous proteins, the well-studied *E. coli* expression systems are still preferred over those of *B. subtilis*. One major reason is that many *Bacillus* plasmid vectors have been reported to exhibit structural instability during replication (Bron *et al.*, 1991). To overcome this problem, integration vectors have been developed allowing stable integration into the chromosome. Another possibility is to use plasmids exhibiting full structural and segregational stability, plasmids allowing improved purification of heterologous proteins, or plasmids containing a strong regulatable promoter and/or an optimized ribosome-binding site (Lam *et al.*, 1998;Kaltwasser *et al.*, 2001;Nguyen *et al.*, 2005; Brockmeier *et al.*, 2006b; Phan *et al.*, 2006; Le and Schumann, 2007; Nguyen *et al.*, 2007).

(III) When a recombinant protein is expressed in bacterial cells, it often accumulates as insoluble aggregates, known as inclusion bodies. Bacterial inclusion bodies are dynamic structures especially formed due to irregular or incomplete folding processes (Ventura and Villaverde, 2006). In order to overcome this limiting factor, the construction of strains that overproduce major intracellular chaperones is one approach to enhance the secretion yield of proteins reducing the formations of insoluble proteins. A previous study reported that overexpression of the two major cytosolic chaperones, DnaK and GroE, increased the secretory production of the antidigoxin single-chain antibody production in *B. subtilis* suggesting that co-overexpression of molecular chaperones decreases aggregation of heterologous proteins and increases their yields (Wu *et al.*, 1998).

(IV) In *B. subtilis*, secretory proteins emerging from the translocon at the cytoplasmic membrane/cell wall interface are directly released into the extracellular environment where a massive amount of proteases are secreted (Bolhuis *et al.*, 1999a). At this point of the secretion process, the first limiting and crucial step is the sufficient and optimal processing of the SP by a SPase (see Topic 1.1). In order to address this problem, a previous study demonstrated that an overexpression of SPase I decreased the limitation in the processing of the mature protein (Van Dijl *et al.*, 1992).

(V) Once the mature protein is released into the "pseudo periplasmic" environment between the cytoplasmic membrane and the cell wall, it needs to be immediately correctly folded into its native conformation. Otherwise, the misfolded proteins are rapidly degraded by the proteases, especially foreign proteins which are more accessible for extracellular proteases than homologous enzymes. To overcome the degradation bottleneck, the use of the engineered *B. subtilis* strains with knockouts of extracellular and/or intracellular proteases has been one successful approach broadly applied. The strains WB600, WB700 and WB800 (deficient in six, seven and eight extracellular proteases, respectively) have been successfully used in the production of some heterologous proteins, which were sensitive to proteolytic degradation (Wu *et al.*, 1991; Wu *et al.*, 2002; Westers *et al.*, 2005; Liu *et al.*, 2008; Liu *et al.*, 2010; Wu *et al.*, 2002).

(VI) The misfolding of heterologous proteins after the translocation due to missing or insufficient chaperone activity is another considerable bottleneck in protein expression. To solve this problem, the overexpression of an extracellular foldase named PrsA, a lipoprotein required for protein stability in the post-translocation stage of secretion, has been suggested to lead to an increase of export yield of heterologous proteins (Kontinen and Sarvas, 1993; Vitikainen *et al.*, 2001; Vitikainen *et al.*, 2005).

(VII) Furthermore, the disulfide bond formation, considered one of the most important processes for the activity and stability of many secreted heterologous proteins, represents another potential bottleneck for the secretion of proteins in *B. subtilis* (Saunders *et al.*, 1987;Bolhuis *et al.*, 1999b). An overexpression of the *B. subtilis* Bdb (*Bacillus* disulfide bond) protein actively involved in the folding of some secretory proteins by catalyzing the formation of disulfide bonds, is claimed to improve secretion of proteins containing possible disulfide bonds (Bolhuis *et al.*, 1999b; Nijland and Kuipers, 2008).

(VIII) Another issue is the composition of the cell wall. After translocation through the cytoplasmatic membrane, the proteins must pass a relatively thick peptidoglycan layer of about 10-50 nm. This layer is negatively charged, thus showing a special affinity to positively charged secretory proteins, causing a delay on the release of secretory proteins. To address this problem, proteins can be engineered to carry specific physico-chemical properties or changes in the net charge of the cell wall can be adapted as described in previous studies (Saunders *et al.*, 1987; Stephenson *et al.*, 1998; Stephenson *et al.*, 2000).

1.4 Objectives of the PhD thesis

The main goal of this PhD project was to construct an efficient secretion system for recombinant proteins in *Bacillus subtilis* using an α-amylase as a reporter enzyme. Overproduction of this enzyme formed aggregates within the cytoplasm or caused the cytoplasmatic membrane to become jammed, leading to partial secretion into the supernatant. In order to increase the amount of secreted α-amylase two experiments were carried out.

First, a co-production and overexpression of SecA was performed by introducing a plasmid coding for overproduction of the *B. subtilis Sec*A.

Second, an artificial *secYEG* operon was constructed composed of *B. subtilis* subunits in order to enhance the amount of translocons in the cytoplasmic membrane.

Another aim of this work was to use the transposon mutagenesis strategy in two different experiments in order to screen for *B. subtilis* mutants able to increase secretion of α-amylase. First, the *mariner*-based transposon was used to detect mutants presenting a higher secretion of α-amylase on indicator plates. Second, transposon mutagenesis of a modified transposon containing a xylose-inducible promoter was carried out to induce or enhance gene expression of gene products that might increase secretion of α-amylase.

An additional experiment was performed to test the *sinIR* transcriptional terminator as a 3' end stabilizing element in an artificial bicistronic operon producing BgaB and GFP as reporter proteins.

2 Materials and methods

2.1 Bacterial strains, plasmids, oligonucleotides, antibiotics, antibodies and media

2.1.1 Bacterial strains and plasmids

The bacteria strains and plasmids used in the course of this work are listed in the Tables 2 and 3.

Table 2: Summary of the bacterial strains used in this work

Name	Description	Reference
E. coli		
DH10B	*str^r F- mcrA* Δ(*mrr hsdRMS mcrBC*) *ϕ80d lacZΔM15* Δ*lacX54 deoR recA1 araD139* Δ(*ara, leu*) *7697 galU galK λ$^-$ rpsL endA1 nupG*	Bethesda Research Laboratories
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F´ proAB lacI^q ZΔM15 Tn10* (Tet^r)]	Stratagene
JM110	*rpsL*(Str^r) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) F` *traD36 proAB lacI^q* ZΔM15	Stratagene
B. subtilis		
1012	*leuA8 metB5 trpC2 hsdRM1*	(Saito *et al.*, 1979)
WB800	*nprE aprE epr bpr mpr*::*ble nprB*::*bsr* Δ*vpr wprA*::*hyg* Cm^r	(Wu *et al.*, 1998)
WB800N	*nprE aprE epr bpr mpr*::*ble nprB*::*bsr* Δ*vpr wprA*::*hyg cm*::*neo* Neo^r	(Nguyen, 2006)

IHA01	1012 with integration of pK2-*spec (lacA::spec)* Spec^r	(Härtl *et al.*, 2001)
KL01	1012 containing both pKL01 and pWKML01	*
KL02	IHA01 with integration of pKL11 (*lacA*::*amyQ*) Erm^r	*
KL03	IHA01 with integration of pKL12 (*lacA*::*secYEG*) Erm^r	*
KL04	1012 with integration of pKL14 (*amyE*:: *i-cat*) Spec^r	*
KL05	1012 with integration of pKL15 (*amyE*:: *cat*) Spec^r	*
KL06	1012 with integration of pKL16 (*th*r*C*:: *i-cat*) Spec^r	*
KL07	1012 with integration of pKL17 (*th*r*C*:: *cat*) Spec^r	*
KL08	1012 with integration of pKL14 (*amyE*:: *i-cat*) Cm^r	*
KL09	1012 with integration of pKL14 (*amyE*:: *i-cat*) Cm^r	*
KL10	1012 with integration of pKL14 (*amyE*:: *i-cat*) Cm^r	*
KL11	1012 with integration of pKL14 (*amyE*:: *i-cat*) Cm^r	*
KL12	1012 containing both pKL01 and pMarA	*

Strains marked with an asterisk (*) were constructed during this work.

Table 3: Summary of plasmids used during this work

Name	Description	Reference
pAX01	Integration plasmid into *lacA* gene with expression xylose cassette	(Härtl *et al.*, 2001)
p*BgaB*	Integration plasmid carrying the promoter-less *bgaB* gene	(Mogk *et al.*, 1996)
pDG1730	Integration vector allowing insertion at the *amyE* locus	(Guérout-Fleury *et al.*, 1996)
pDG1731	Integration vector allowing insertion at the *thrC* locus	(Guérout-Fleury *et al.*, 1996)

pHCMC01	pMTLBs72 with *trpA* transcriptional terminator	(Nguyen, 2006)
pHCMC05	pHCMC01 with IPTG-inducible, Pspac promoter	(Nguyen, 2006)
pHT01	Expression vector with the IPTG-inducible P_{grac} promoter	(Phan *et al.*, 2006)
pHT212	pHCMC01 with *bgaB* fused to the P_{groES} promoter	(Nguyen *et al.*, 2007)
pHT43	pHT01 with *amyQ* signal sequence fused to the SD sequence	(Phan *et al.*, 2006)
pHTJM	*gfp* inserted into pHT01	(Martini, 2009)
pKL01	pHT212 with *bgaB* replaced by *amyQ*	*
pKL02	*bgaB* inserted into pHT01	*
pKL03	*gfp* inserted downstream of *bgaB* into pKL02	*
pKL04	*sinIR* terminator inserted between *bgaB* and *gfp* into pKL03	*
pKL05	*trpA* terminator inserted between *bgaB* and *gfp* genes in pKL03	*
pKL06	pHT01 with IPTG-inducible P*spac* promoter replaced by P*grac* promoter	*
pKL07	*secG* inserted into pHCMC01	*
pKL08	*secE* inserted into pKL07	*
pKL09	*secY* inserted into pKL08	*
pKL10	*secYEG* inserted into pKL06	*
pKL11	*amyQ* inserted into pAX01	*
pKL12	*secYEG* inserted into pAX01	*
pKL13	Xylose cassette inserted into pMarA	*
pKL14	*cat* gene inserted into pDG1730 in one orientation	*

pKL15	*cat* gene inserted into pDG1730 in the inverse orientation	*
pKL16	*cat* gene inserted into pDG1731 in one orientation	*
pKL17	*cat* gene inserted into pDG1731 in the inverse orientation	*
pKTH10	*amyQ* inserted into pUB110	(Palva, 1982)
pMarA	TnYLB-1 delivery plasmid with a *mariner-Himar1* transposase	(Le Breton *et al.*, 2006)
pMTLBs72	*E. coli*/*B. subtilis* shuttle vector, the backbone for the series of pHCMC an pHT vectors	(Titok *et al.*, 2003)
pNDH37-*celA*	pNDH37 with mature part of *celA* gene	(Nguyen, 2006)
pWKML01	pWH1520 with *secA* under the control of a *xylA* promoter	(Leloup *et al.*, 1999)
pX	Xylose inducible integration vector	(Kim *et al.*, 1996)

Strains marked with an asterisk (*) were constructed during this work.

2.1.2 **Oligonucleotides**

The oligonucleotides used during this work are listed in the Table 4. All oligonucleotides were obtained as high purified, salt free and lyophilized. The primers were dissolved in distilled water yielding a final concentration of 100 pmol/µl and stored at –20°C.

Table 4: Summary of oligonucleotides used in the course of this work

Name	Sequence 5' to 3'	Description
ON01	GGCCATGGATCCATGATTCAAAAACGAAAGCGGA	5' end of *amyQ* gene
ON02	GGCCATGACGTCTTATTTCTGAACATAAATGGAGA	3' end of *amyQ* gene

ON03	CGTGGGGAAGGAAAAGCGTGGGATT	*amyQ* gene from 619bp to 643bp
ON04	CTAATACGACTCACTATAGGGAGATCCTTGTGAGGAAGCCGACTGTAAAT	3’ end of *amyQ* gene with T7 promoter sequence
ON05	GGCCATTCTAGACGGCCGTACGGCTGATGTTTTTG	3' end of *amyQ* signal sequence for insertion in pHT10
ON06	GGCCATGGATCCATGAATGTGTTATCCTCAATTTGT	5' end of *bgaB* gene
ON07	GGCCATGGATCCCCGCGGCTAAACCTTCCCGGCTTCATCATG	3' end of *bgaB* gene
ON08	GGCCATGACGTCAGAAAGGAGGTGATCATGAGCAAAGGAGAAGAA	5' end of *gfp* gene
ON09	GGCCATCCCGGGTTATTTGTAGAGCTCATCCATG	3' end of *gfp* gene
ON10	GGCCATTCTAGATCCCAAAAAGAGGAGTAGTG	5' end of *sinIR* transcriptional terminator
ON11	GGCCATGACGTCACTAGTTTCGAAGCTACACAGTGGAACGGCT	3' end of *sinIR* transcriptional terminator
ON12	CTAGAGCAGCCCGCCTAATGAGCGGGCTTTTTTACTAGTGACGT	5' end of *trpA* transcriptional terminator
ON13	TCGTCGGGGCGGATTACTCGCCCGAAAAAATGATCAC	3' end of *trpA* transcriptional terminator
ON14	GGCCATGAGCTCAGCTATTGTAACATAATCGGTACG	5' end of P*grac* promoter
ON15	GGCCATGGATCCTTCCTCCTTTAATTGG	3' end of P*grac* promoter
ON16	GGCCATGGTACCAGGCCTTACACAGCCCAGTCCA	5' end of P*spac* promoter
ON17	GGCCATGGATCCTCACCTCCTTAAGCTTAATTGT	3' end of P*spac* promoter
ON18	GGCCATTCTAGAAAAGAAGCTGTTGAAGCTGCTGGC	5' end of *secY* gene

ON19	GGCCATGGATCCCTAGTTTTTCATAAATCCACGGTA	3' end of *secY* gene
ON20	GGCCATGGATCCATCTTGAAACAAAATAGTTTTTGC	5' end of *secE* gene
ON21	GGCCATGGTACCTTATTCAACTATTAAACGAATTAA	3' end of *secE* gene
ON22	GGCCATGGTACCTTCATGTAAAATAGAAGTAATGTA	5' end of *secG* gene
ON23	GGCCATGAGCTCCTATAGGATATAAGCAAGCGCAAT	3' end of *secG* gene
ON24	GGCCATTCTAGAAAAGAAGCTGTTGAAGCTGCTGGC	5' end of *secYEG* gene
ON25	GGCCATACTAGTGCTGAGGTGATCTAACATGTTTA	5' end of *secYEG* gene
ON26	GGCCATACTAGTCTCCTATAGGATATAAGCAAGCG	5' end of *secYEG* gene
ON27	GGCCATGACGTCCTATAGGATATAAGCAAGCGCAAT	3' end of *secYEG* gene
ON28	ATGCGTATTATGAAATTCTTTAAAGATG	5' end of *secE* gene
ON29	CTAATACGACTCACTATAGGGAGACTATAGGATATAAGCAAGCGCAATC	3' end of *secG* gene
ON30	AGTAAAGTTATCGGAATCGACTTAG	5' end of *dnaK* gene
ON31	CTAATACGACTCACTATAGGGAGAAAAGTATGCAGGAACTGTGTA	3' end of *dnaK* gene
ON32	GGCCATGGGTCCCCGGCCGCTAACTTATAGGGGTAACACTTAAAA	5' end of *xyl* repressor and promoter genes
ON33	GGCCATGGGACCCCATTTCCCCCTTTGATTTTTAGAT	3' end of *xyl* repressor and promoter genes
ON34	GGCCATGGATCCTAGATAAAAATTTAGGAGGCATATCAAATG	5' end of *cat* gene
ON35	GGCCATAAGCTTTCTCATATTATAAAAGCCAGTCATTAGGCC	3' end of *cat* gene

ON36	GGCCATAAGCTTAATTTAGGAGGCATATCAAATGAACTTT	5' end of *cat* gene
ON37	GGCCATGGATCCTTATAAAAGCCAGTCATTAGGCCTATCTG	3' end of *cat* gene
ON38	GGCCATTAAAAATCAAAGGGGGAAATG	5’ end of *xylA* gene
ON39	GGCCATATTTAGGAGGCATATCAAATG	3’ end of *i-cat* gene
ON40	GGCCATCATTTGATATGCCTCCTAAAT	5’ end of *cat* gene

ON, oligonucleotide; the DNA sequences recognized by restriction enzymes are underlined

2.1.3 **Antibiotics**

The Table 5 gives the information concerning the antibiotics solutions used in the course of this work. The antibiotics were obtained in p.a. quality from the following companies: Serva and Sigma-Aldrich.

Table 5: Summary of antibiotic solutions used in this work

Antibiotic	Concentration of stock solution (mg/ml)	Dissolved in	Final concentration (µg/ml)
Ampicillin	100	water	100
Chloramphenicol	100	ethanol	10
Erythromycin	1 or 100	ethanol	1 or 100
Kanamycin	100	Water	100
Neomycin	10	water	10
Spectinomycin	100	Water	100
Tetracycline	10	10 % ethanol	100

2.1.4 **Antibodies**

Table 6: Summary of antibodies used in the course of this work

Name	From (organism)	Dilution	Second antibody
α-AmyQ	*B. amyloliquefaciens*	1:15.000	Rabbit
α-CelA	*C. thermocellum*	1:10.000	Rabbit

α-TrxA	*B. subtilis*	1:1.000	Rabbit
α-SecA	*B. subtilis*	1:10.000	Rabbit
α-SecY	*E. coli*	1:5000	Rabbit
α-DnaK	*B. subtilis*	1:5000	Rabbit
α-FtsH	*B. subtilis*	1:5000	Rabbit

2.1.5 **Media**

LB medium (Luria Bertani): 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl.

SRM (Super Rich Medium): 1.5 % (w/v) tryptone, 2.5 % (w/v) yeast extract, 0.3 % (w/v) K_2HPO_4 and 1 % (w/v) glucose (143).

Antibiotics (Table 5) and 1 % (w/v) insoluble starch were added when necessary.

Agar was added to 1.5 % (w/v) to prepare plates.

2.2 Enzymes, biochemicals, chemicals and kits

2.2.1 **Enzymes**

Roche: alkaline phosphatase, T7 RNA polymerase and DNase I

Merk: proteinase K

Sigma-Aldrich: RNase A and lysozyme

Fermentas: restriction enzymes, T4 DNA ligase and *Taq*-DNA polymerase

Stratagene: Pfu-DNA polymerase

2.2.2 **Biochemicals and chemicals**

Amersham: Amonium persulphate, hyperfilm ECL

Fermentas: DNA ladder, RNA ladder and Prestained and Unstained protein molecular weight markers

Pierce: Luminol substrate

Roche: blocking reagent, chemiluminescent substrate CPD-Star, protease inhibitor, RNAase inhibitor, Xgal, and ONPG

Thermo Scientific: Super Signal West Pico Chemiluminescent substrate

Roth: acetic acid, agar, agarose, aqua phenol, chloroform, DEPC, ethidium bromide, isopropanol, pepton, potassium acetate, potassium phosphate, polyacrylamide, sodium phosphate, sodium chloride, starch, MOPS, sodium dodecyl sulphate, sucrose, Tris, xylose and yeast extract

2.2.3 **Kits**

Qiagen: gel-extraction and midi purification kits
Peqlab: PCR purification kit

2.3 General methods

2.3.1 **PCR**

PCR (Polymerase Chain Reaction) was carried out in a total volume of 50 µl containing 20–50 ng of template DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer, and 1 unit of Pfu or Taq-DNA polymerase Amplifications were performed using a thermocycler (BioRad) with the following conditions: one cycle of 95^{o}C for 5 min, 35 cycles of 95^{o}C for 1 min, 55 – 60^{o}C for 30 sec. or 1 min (according to each primer) and 72^{o}C for 1 min, and one cycle of 72^{o}C for 20 min of final extension.

Colony PCR was performed after ligation based on standard PCR (following the conditions above) using cells from single colonies (total DNA) as template allowing rapid detection of the putative positive clones by detection of the amplified insert.

Complementary oligonucleotides were annealed using 5 µl of each oligonucleotide (100 pmol/µl) diluted into 20 µl 1X SSC buffer. The reaction was heated at 75^{o} C for 5 min and kept at room temperature for 3 h. The PCR as well as the complementary oligonucleotide products were treated with the appropriated enzymes for preparing the DNA sequence for the ligation and transformation steps.

2.3.2 **Cloning**

Cloning steps were carried out as previously described (Sambrook and Russell, 2001). Preparation of competent *E. coli* cells and transformation were performed either by standard heat shock transformation (Inoue *et al.*, 1990) or electroporation (Dower *et al.*, 1992). The procedure for extracting and preparing plasmid DNA followed the alkaline lysis method (Birnboim and Doly, 1979). The correct DNA sequence of all plasmids was verified by restriction enzyme analyses using at least five different enzymes and only plasmids showing the expected fragments were transformed into *B. subtilis* strains and used for further experiments. The method described by Anagnostopoulos and Spizizen was used for the preparation of competent cells and transformation in *B. subtilis* strains (Anagnostopoulos and Spizizen, 1961).

2.3.3 **Growth and collection of samples**

B. subtilis strains were grown in either LB or SRM medium in a water bath shaker at 200 rpm at 37 or 30°C with the appropriate antibiotic(s). Cultures of 4 ml medium grown overnight in glass tubes were transferred to Erlenmeyer flasks containing medium and appropriate antibiotics to reach an OD_{578} of 0.08. At OD_{578} of 0.8 (set as t = 0) culture was divided into two subcultures where one was further grown in the absence and the other in the presence of the appropriate inducer (0.1 mM or 1 mM of IPTG and/or 0.25 or 0.5% xylose). Aliquots were collected and

separated into pellet and culture supernatant by centrifugation. Further samples were taken at different time points according to the objective of each experiment.

2.4 RNA: Northern blot analysis

Northern blot analysis was carried out to confirm the quality and quantity of mRNA and performed as previously described (Homuth *et al.*, 1997).

2.4.1 Isolation of total RNA from *B. subtilis*

Total RNA was extracted using the protocol for isolation of RNA from yeast with modifications (Robert, 1998). *B. subtilis* cells were grown and induced as described in 2.3.3. For the analyses of the *amyQ* transcript the cells were collected at an OD_{578} of 1.0. For the analyses of *secEG* transcripts the cells were collected at the OD_{578} of 0.8, 1.5, 2.5 and 3.5 (set as t = 0, 1, 2 and 3, respectively). The cells were killed by addition of "killing buffer" (5 mM $MgCl_2$, 20 mM NaN_3, 20 mM Tris-HCl - pH7.5). The cell wall was then digested with "lysis buffer" (25 % sucrose, 20 mM Tris-HCl – pH8.0, 0.25 mM EDTA) and lysozyme (1 mg/ml) on ice for 5 min. To lead to the lysis of the protoplasts the samples were heated at 95 °C for 5 min and then treated with phenol-chloroform.

2.4.2 Electrophoresis of RNA and vacuum blot transfer to membranes

RNA samples were separated by size via electrophoresis in a 1.2% agarose gel under denaturing conditions. The transfer was performed onto a nylon membrane and carried out with a Vacuum-Blot-Annex (VacuGene^{TM}X1, Pharmacia).

2.4.3 Transcriptional labeling of RNA probes

The pairs of primers ON03/ON04 and ON28/ON29 were used to amplify part of the *amyQ* gene from pKL01 and the *secE* and *secG* genes from pKL10, respectively. The pair of primers ON30/ON31 was used to amplify the *dnaK* gene here used as a loading control. These amplicons containing a T7 promoter at the 3' primer end was used as labeled probe for crosslinking and hybridizing *in vitro.* The *in vitro* transcription was performed according to the instructions of the manufacturer (Roche).

2.4.4 **Hybridization of membrane-bound RNA with RNA probes**

This experiment was carried out as described (Roche)

2.4.5 **Stripping of RNA probes**

This experiment was carried out as described (Roche)

2.5 Protein: Western blot analysis

2.5.1 **Preparation of soluble and insoluble cell extracts from *B. subtilis***

To examine the solubility of α-amylase produced in the cytoplasm, cells were disrupted on ice by ultrasonication (12 W, 6 x 15 pulses with 15 sec intervals) in an 1.5 ml Eppendorf tube containing 1 ml of cell suspension (100 mM NaCl, 50 mM Tris-HCl – pH 7.5 and Proteinase Inhibitor). Afterwards, 50 µl of the preparation were collected for whole cell extract and the cells were centrifuged at 5.000 rpm for 15 min to remove cell debris. Subsequent centrifugation of the supernatant at 45.000 rpm for 1½ h separated the insoluble (membrane) and soluble (cytoplasmic) protein fractions. The amount of protein corresponding to 10 µg/µl was separated by SDS-PAGE, followed by immunoblotting with specific antiserum. The method of Bradford was used for the measurement of the protein concentrations from cell extracts (Kruger, 2002).

2.5.2 **Determination of protein concentration**

The protein concentration was measured spectrophotometrically at 595 nm according to the method of Bradford (Kruger, 2002) using BSA as the standard.

2.5.3 **Precipitation of proteins from culture supernatants**

Protein from cultured supernatant was collected by the TCA precipitation method. One volume of culture was precipitated in 40% TCA to reach a final concentration of 10% and incubated on ice for 30 min, then centrifuged at 12.000 rpm at 4° C for 10 min. The pellet was washed twice with ice-cold acetone and dried at room temperature. Then, it was dissolved in 50 µl water and 25 µl 3X loading buffer (Laemmli, 1970). For Western blot analysis the suspension was diluted 15-fold and the proteins were separated by SDS-PAGE, followed by immunoblotting with specific antiserum.

2.5.4 **Western blot analysis**

In order to detect proteins immunochemically, the proteins were first separated by SDS-PAGE and then transferred under semi-dry conditions onto either a nitrocellulose or a polyvinylidene difluoride (PVDF) membrane using electroblotting (Towbin *et al.*, 1979). The electrophoretic transfer of proteins was performed in a "Fast-Blot" apparatus (Biorad) and carried out for 15 min at 13 V, 3 A and 300 mA using Blotting buffer (3 g Tris, 14 g glycine, 20% (v/v) methanol, 0.1% (w/v) SDS). After the transfer, blocking of the membrane was achieved by incubation in AP-T buffer (1 M Tris/HCl pH 7.4, 1 M NaCl, 25 mM $MgCl_2$, 0.03% (v/v) Tween 20) containing 5% (w/v) milk powder. The following steps were then carried out only in AP-T buffer. The procedure for detection of labeled proteins followed the instruction of LAS4000 (FUJIFILM Life Science - USA). For additional information about the antibodies used in all immunoblot analyses in this work see Table 6.

2.6 Visualization and measurement of reporter gene expression

2.6.1 **Visualization of extracellular enzyme activity of α-amylase on plates**

Single colonies of the *B. subtilis* strain 1012/pKL01 were grown for 16 h on LB plates containing different concentrations of IPTG and 1% soluble starch and stained with I_2/KI solution (Nicholson and Chambliss, 1985). The α-amylase activity was indicated by yellow halos around the colonies.

2.6.2 **Measurement of the α-amylase activity**

The strain 1012/pKL01 was grown and induced in LB medium at 37°C as described under 2.3.3. 24 h aliquots were collected and the α-amylase activity was determined as previously descrbed (Nicholson and Chambliss, 1985). Briefly, 250 µl of diluted supernatant was added to 1 ml of buffer with soluble starch (50 mM Tris/HCl pH 6.8, 25 mM $CaCl_2$ and 0.05% soluble starch) and 0.1% I_2/KI was used to stop the reaction. α-amylase activity was determined by the decrease in absorbance at OD_{620} of 0.1, defined as 1 U of activity.

2.6.3 **Measurement of the β-galactosidase activity**

Blue colonies from LB-Xgal plates (50 µl 4% Xgal per plate) were used for determination of β-galactosidase activity. *B. subtilis* strains 1012/pKL02, 1012/pKL03 and 1012/pKL04 were grown at 37°C and induced with 1 mM IPTG, as described in 2.3.3. 10 ml samples were collected at t = 0 (before induction) and at t = 2, 4 and 6 h. The activity was determined at 55°C as previously described (Mogk *et al.*, 1996). One unit is defined as $\Delta A_{420}*OD_{578}^{-1}/min^{-1}$ and displayed as units/OD_{578} for all the results, in which one OD_{578} is defined as the optical density of the samples used in the assay, A_{420} is the absorbance of the samples measured by the reader and min indicates the incubation time of the plate at 55° C.

2.6.4 **Microscopy and GFP fluorescence analysis**

In order to guarantee that the plasmids pKL03 and pKL04 were successfully transformed into the *E. coli*, single colonies of these strains were grown for 24 h on LB plates containing 1 mM IPTG.

These plates were observed under a MZFLIII microscope (Leica) using the GFP2 filter. For analysis of the GFP fluorescence by the vectors described above transformed either into *E. coli* or *B. subtilis*, the software Image Reader LAS-4000 was used (FUJIFILM Life Science - USA).

2.7 Constructions of the plasmids and strains

2.7.1 Construction of terminator-test vectors

In order to construct a terminator-test vector allowing the identification of transcriptional terminators including 3' stabilizing elements, the plasmid pHT01 was used as a backbone vector and the BgaB and GFP were used as reporter proteins. The *bgaB* gene was amplified with its respective start and stop codons by PCR using the primers ON06 and ON07 and the plasmid pBgaB as a template. The amplicon was cleaved with *Bam*HI and cloned downstream the P*grac* promoter region into pHT01 resulting in the pKL02 (Fig. 6 A). The correct orientation was determined by an extra cleavage in the *Sac*II restriction site present in the 3' primer. This vector was used as a positive control to test the activity of BgaB and also as a negative control to verify the fusion and expression of GFP. The *gfp* gene was amplified with its respective start and stop codons by PCR using the primers ON08 and ON09 and the plasmid pHTJM as a template. The product was cleaved with *Aat*II and *Sma*I and cloned downstream the *bgaB* gene resulting in the pKL03 (Fig. 6 B).

The *sinIR* transcriptional terminator was cloned between both reporter genes to ensure efficient termination of transcription immediately downstream of the *bgaB* gene (Fig. 6 C). The *sinIR* DNA sequence corresponding to the structure of two terminators located at the end of the *sin* operon in *B. subtilis* was constructed using the complementary oligonucleotides ON10 and ON11. The product was cleaved with *Xba*I and *Aat*II and cloned between *bgaB* and *gfp* genes into pKL03 resulting in the pKL04. The effect of this transcriptional terminator was analyzed in both *E. coli* and *B. subtilis* by the measurement of the β-galactosidase activity and the GFP fluorescence analysis.

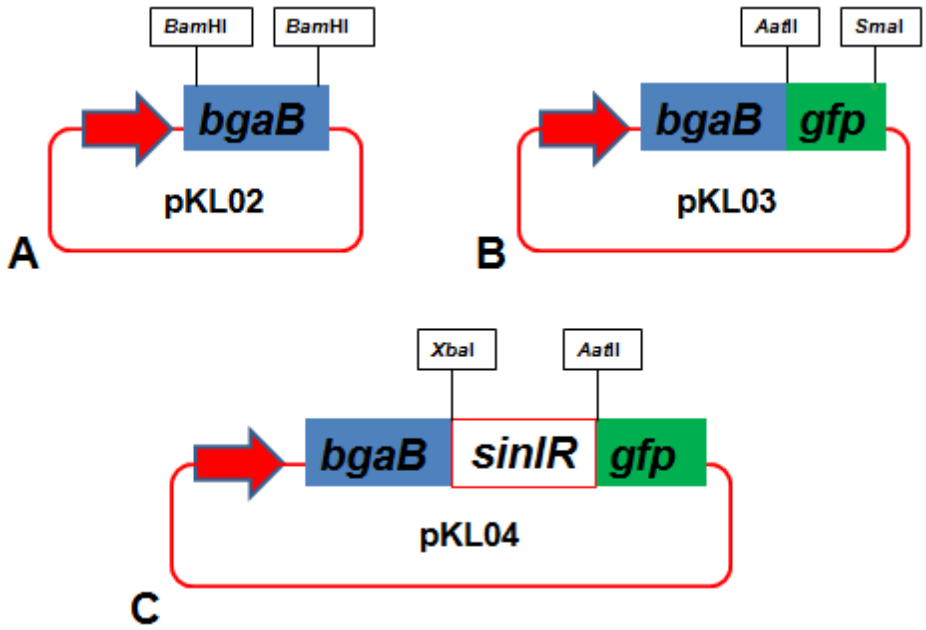

Figure 6: Terminator–test vector
The restriction sites used for insertion of the DNA sequences are shown in the white boxes. Arrow: IPTG-inducible P_{grac} promoter. (**A**) *bgaB* gene fused to pHT01 downstream of P*grac* promoter. **B**) *gfp* gene fused downstream of *bgaB* gene. (**C**) *sinIR* transcription terminator inserted between *bgaB* and *gfp* genes.

2.7.2 Vectors and strains for the overexpression of α-amylase

The vector pKL01 was constructed in order to analyze secretion of the protein α-amylase in *B. subtilis*. The *amyQ* gene was amplified by PCR using the primers ON01 and ON02 and the plasmid pKTH10 as a template. The amplicon was cleaved with *Bam*HI and *Aat*II and cloned into the plasmid pHT212 under the control of the IPTG-inducible P_{groES} promoter. The *amyQ* replaced the *bgaB* gene. Two different approaches were carried out to verify the amount of protein released by the cells into the supernatant compared to the remaining protein either inside the cytoplasm or attached to the membrane.

2.7.2.1 Overexpression of SecA protein

In order to verify whether the overexpression of SecA can increase secretion of α-amylase in *B. subtilis* cells, the vector pWKML1 (Leloup *et al.*, 1999) which bears the *secA* wild-type gene of

B. subtilis 168 under the control of a xylose-inducible promoter was transformed into the strain 1012/pKL01 resulting in the strain KL01 (pKL01 + pWKML01). The strain 1012/pKL01 was used as control.

Both strains were grown in LB medium at 37°C and induced with either 0.25 or 0.5% xylose and 0.1 mM IPTG at an OD_{578} of 0.8. Samples were collected after 12 and 24 h. The synthesis of SecA in both strains was analyzed by Western blot using antibodies raised in rabbits against SecA at a dilution of 1:10.000. To analyze overexpression and secretion of α-amylase, 12 and 24 h samples were fractioned into cytoplasmic, membrane and supernatant fractions. A final concentration of 10 µg/µl of each fraction was loaded onto a SDS-PAGE gel and Western blot experiment were carried out using antibodies at a dilution of 1:20.000.

2.7.2.2 Construction of an artificial *secYEG* operon

The second approach to achieve overproduction and secretion of α-amylase in *B. subtilis* cells was based on the construction of an artificial *secYEG* operon in order to enhance the amount of translocons in the cytoplasmic membrane. For this purpose, the genes *secY*, *secE* and *secG* containing their respective Shine-Dalgarno sequence, start and stop codons were amplified from *B. subtilis* 1012 chromosomal DNA by PCR using the pairs of primers ON18/ON19, ON20/ON21 and ON22/ON23, respectively.

The first step was to construct the plasmid pKL06 where the P_{grac} promoter was replaced by the IPTG-inducible P_{spac} promoter. The P_{spac} promoter was amplified by PCR using the primers ON16 and ON17 and the plasmid pHCMC05 as a template. The amplicon was treated with *Kpn*I and *Bam*HI and cloned into pHT01.

Secondly, *secY*, *secE* and *secG* genes were individually inserted into the plasmid pHCMC01 to construct the artificial *secYEG* operon. The *secG* amplicon was cleaved with *Kpn*I and *Sac*I and cloned into pHCMC01 resulting in the plasmid pKL07. The *secE* amplicon was treated with *Kpn*I and *Bam*HI and cloned into pKL07 resulting in the plasmid pKL08. Then, the *secY* product

was cleaved with *Bam*HI and *Xba*I and inserted into pKL08 resulting in the plasmid pKL09 (Fig. 7 A).

The sequence comprising the *secYEG* operon was excised from pKL09 using the *Xba*I and *Aat*II restriction sites and inserted into the plasmid pKL06 under the control of the IPTG-inducible P*spac* promoter, resulting in the plasmid pKL10 (Fig. 7 B).

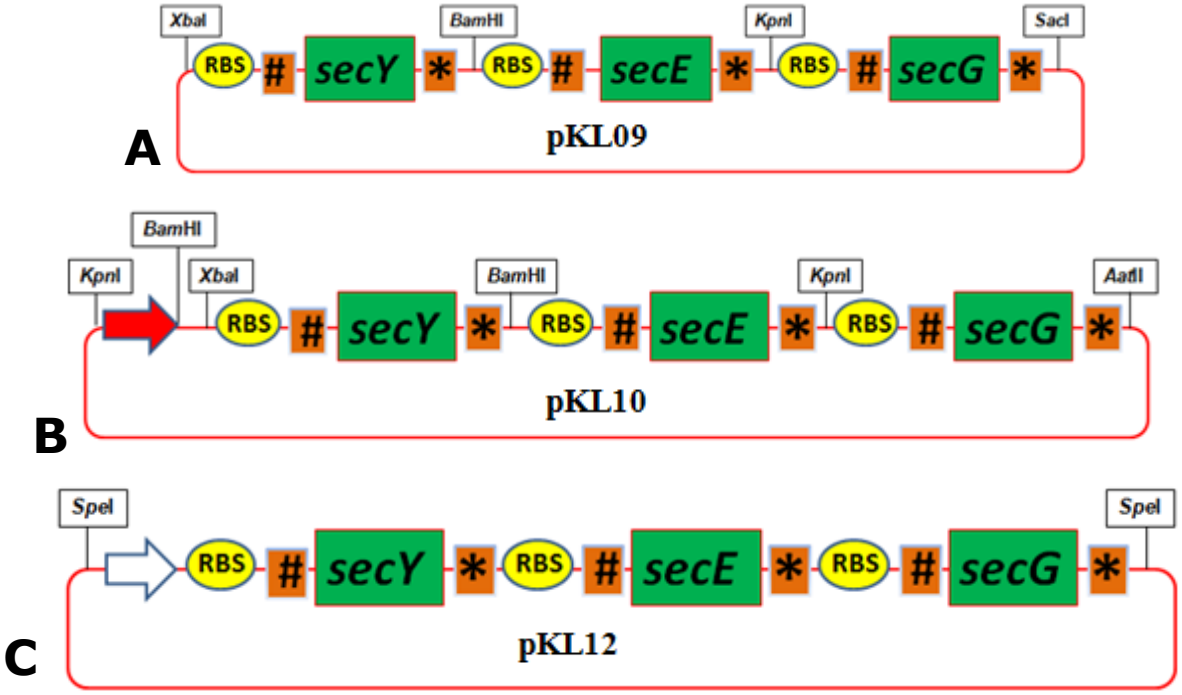

Figure 7: Construction of an artificial secYEG operon
The restriction sites used for insertion of the genes are shown in the white boxes. RBS: ribosome binding site; #: start codon; *: stop codon; red arrow: IPTG-inducible P_{spac} promoter; white arrow: xylose-inducible P_{xylA} promoter. (**A**) *secY*, *secE* and *secG* genes fused into the plasmid pHCMC01 (without promoter) containing their Shine-Dalgarno sequence, start and stop codons. (**B**) *secYEG* genes from pHCMC01 inserted into pHT01 under the control of the ITPG-inducible P_{spac} promoter. (**C**) *secYEG* genes fused to the delivery plasmid pAX01 under the control of a xylose-inducible P_{xylA} promoter and integrated into the bacterial chromosome via a double crossing-over event at the *lacA* locus.

Here, two different approaches were carried out in order to analyse secretion of α-amylase.

First, the *amyQ* gene was integrated into *B. subtilis* strain IHA01 (*lacA::spec*) resulting in the strain KL02 (*lacA::amyQ*). The *amyQ* gene was amplified by PCR using the plasmid pKL01 as a

template. The amplicon was cleaved with *Bam*HI and *Sac*II and fused to a xylose-inducible P_{xylA} promoter present in the delivery plasmid pAX01, resulting in the plasmid pKL11, which was then transformed into IHA01 *(lacA::spec*) and integrated ectopically into the bacterial chromosome via a double crossing-over event at the *lacA* locus (strain KL02). Colonies presenting the appropriate phenotype, i.e., Erm^r and $Spec^s$, were used as receptor strain for transformation of the plasmid pKL10. Both *amyQ* and *secYEG* genes were induced with 0.5 % xylose and 0.1 mM IPTG at an OD_{578} of 0.8, respectively. Samples were collected after 12 and 24 h and fractioned into cytoplasmic, membrane and supernatant fractions. The samples were analyzed by Western blot using antibodies at a dilution of 1:20.000.

In the second experiment, the *secYEG* operon was integrated into *B. subtilis* strain IHA01 (*lacA::spec*) resulting in the strain KL03 (*lacA::secYEG*). The *secYEG* was amplified by PCR using the plasmid pKL09 as a template. The amplicon was cleaved with *Spe*I and fused to the delivery plasmid pAX01, resulting in the plasmid pKL12 (Fig. 7 C). The correct orientation was determined by an extra cleavage in the *Eco*RV restriction site. The plasmid pKL12 was then transformed into IHA01 *(lacA::spec*) and integrated ectopically into the bacterial chromosome via a double crossing-over event at the *lacA* locus resulting in the strain KL03. Colonies presenting Erm^r and $Spec^s$ were used as receptor strain for transformation of the plasmid pKL01. Both *amyQ* and *secYEG* genes were induced with 0.1 mM IPTG and 0.5 % xylose at an OD_{578} of 0.8, respectively. Samples were collected and analyzed as described above. The strain IHA01/pKL01 was used as control.

2.8 Transposon mutagenesis and construction of a modified transposon

Two different experiments using the transposon mutagenesis strategy were carried out in order to screen for *B. subtilis* mutants able to increase secretion of α-amylase. In the first experiment, transposon mutagenesis was carried out to inactivate gene(s), whose product might regulate directly or indirectly the secretion of α-amylase. This should result in enhanced halos on indicator plates. In the second experiment, I devised a modified transposon containing a xylose-expression cassette which might lead to increased production of a gene product in the presence

of xylose. This gene product might then enhance secretion of α-amylase to be detected by the same technique described for the first experiment.

2.8.1 **Detection of mutants able to increase secretion of α-amylase**

In the first experiment, transposon mutagenesis was carried out using the plasmid pMarA, a transposon-delivery plasmid consisting of the *mariner*-based transposon, TnYLB-1, and a *mariner-Himar*1 transposase gene under the control of σA-dependent promoter (Fig. 8 A) (Le Breton *et al.*, 2006). The plasmid was transformed into *B. subtilis* strain 1012. Transformants were selected on plates containing Kan^r (5 μg/ml) at 30 °C for 36 h, and then screened for plasmid-associated properties, i.e., Kan^r and Erm^r (1 μg/ml) at the permissive temperature allowing plasmid replication (30 °C).

Representative plasmid-containing colonies was used as a receptor strain and the plasmid pKL01 was then transformed, generating the strain KL12 (1012 containing both pKL01 and pMarA). Transformants were selected on plates containing Kan^r and Cm^r (10 μg/ml) at 30 °C for 36 h. Transposon mutagenesis was carried out as follow. An isolated clone selected at Kan^r and Cm^r at 30 °C was grown overnight in liquid LB medium at 30 °C. An aliquot of the overnight culture was used to inoculate 50 ml LB medium at an OD_{578} of 0.08. The culture was grown until an OD_{578} of 1.0 was reached and it was switched to 50 °C for additional ~ 5 h. Transposon mutants were selected at 37 °C. The resulting α-amylase activities were analyzed on indicator LB plates containing 1 % starch.

2.8.2 **Construction of a modified transposon**

In the second experiment, I devised a transposon which can induce or enhance expression when transposed upstream of the gene. Here, the xylose-expression cassette was inserted near one end

of the transposon. In order to construct the modified transposon, the plasmid pMarA was used as backbone. The xylose-expression cassette was amplified by PCR using the primers ON32 and ON33 and the plasmid pX as template. The product was cleaved with *San*DI and inserted into the plasmid pMarA, resulting in the plasmid pKL13 (Fig. 8 B).

The xylose-expression cassette was inserted into the unique *San*DI restriction site ~100 bp upstream of the TnYLB-1 transposon, where the Kan^r cassette is bracketed by one of the *Himar1*-recognized indirect terminal repeats. The correct orientation was determined by an extra cleavage in the *Eag*I restriction site present in the 5' primer. The plasmid pKL13 was transformed into *B. subtilis* strain 1012, and transposon mutagenesis was carried out as described above.

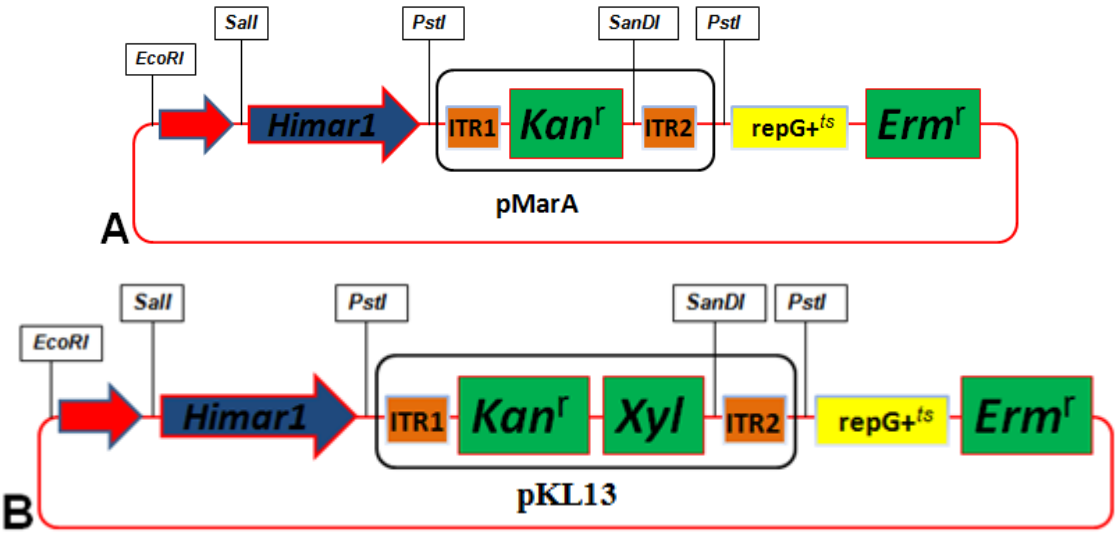

Figure 8: Construction of a transposon-delivery plasmid carrying a xylose-inducible promoter
A) pMarA; **B**) pMarA-xyl containing the xylose expression cassette inserted into the transposable element TnYLB-1. The restriction sites used for insertion of the *cat* gene are shown in the white boxes; red arrow: σA-dependent promoter; *Himar*1: transposase gene; ITR: Inverse terminal repeat; Kan^r: Kanamycin resistance marker; Xyl: xylose expression cassette; repG+^{ts}: origin of replication thermo sensitive; Erm^r: erythromycin resistance marker.

2.8.2.1 The *cat* gene as reporter gene

To verify whether the transposition events might allow or improve expression of genes in *B. subtilis* due to the presence of a xylose-inducible promoter, the promoter-less chloramphenicol

resistance marker (*cat* gene - chloramphenicol acetyltransferase) was used as a reporter gene. Without its own promoter the *cat* gene was inserted in two orientations into both integration vectors pDG1730 and pGD1731.

The *cat* gene was amplified by PCR using the primers ON34 and ON35 and the plasmid pKL01 as template. The amplicon was treated with *Bam*HI and *Hind*III and cloned into the vectors pDG1730 and pGD1731, resulting in the plasmids pKL14 and pKL16, respectively. The inverted *cat* gene was amplified by PCR using the primers ON36 and ON37 and treated with the same enzymes described above. The cloning of the inverted *cat* gene into the vectors pDG1730 and pGD1731 resulted in the plasmids pKL15 and pKL17, respectively.

The plasmids pKL14/pKL15 and pKL16/pKL17 were individually transformed into *B. subtilis* strain 1012 and integrated ectopically into the bacterial chromosome via a double crossing-over event at the *amyE* and *thrC* loci, resulting in the strains KL04/KL05 (*amyE*::*cat/amyE*::*i-cat*) and KL06/KL07 (*th*r*C*::*cat/th*r*C*::*i-cat*), respectively. Colonies presenting appropriate phenotype, i.e., $Spec^r$ and Erm^s, were used as receptive strains for transformation of the plasmid pKL13.

2.8.2.2 Mapping and sequencing of transposon insertion sites

Genomic DNA from transposon mutants was extracted and amplified by PCR using the primers ON38 and ON39 for chromosomal DNA isolated from the strain KL05/pKL13; and the primers ON38 and ON40 for DNA isolated from the strains KL04/pKL13 and KL06/pKL13. 50 or 125 ng of DNA (depending on the PCR fragment) was added to a reaction containing 100 pm/µl of one primer. In order to map the transposon insertion site, the DNA sequences was verified by sequencing carried out by SeqLab. The WebLogo program (http://www.bio.cam.ac.uk/cgi-bin/seqlogo.cgi) was used to align the DNA sequence around the *mariner* insertion sites (Schneider and Stephens, 1990).

3 Results

This work is divided into two parts. The first part consists of the construction of a terminator-test vector. For this purpose, *sinIR* sequence was used as transcriptional terminator to verify whether this sequence act as 3'-stabilizing element of the mRNA, enhancing expression of an upstream gene. An artificial bicistronic operon producing BgaB and GFP as reporter proteins was constructed. The experiments were carried out in both *E. coli* and *B. subtilis.*

The second and main part of this work was focused on the construction of an efficient secretion system allowing hypersecretion of recombinant proteins in *B. subtilis* using the α-amylase protein as a reporter enzyme. The plasmid pKL01 was constructed and used in three different approaches. The first goal was to verify whether the overexpression of SecA of *B. subtilis* can increase secretion of α-amylase. The second goal was to achieve overproduction and secretion of α-amylase in *B. subtilis* cells based on the construction of an artificial *secYEG* operon in order to enhance the amount of translocons in the cytoplasmic membrane.

Last, two different experiments using the transposon mutagenesis strategy were performed. The first experiment was based on a transposon-based strategy for inactivation of genes that might increase secretion of α-amylase using the *mariner*-based transposon. In the second experiment, a modified transposon containing a xylose-expression cassette was constructed for induction of expression of genes whose might be related to the secretion of proteins in *B. subtilis*.

3.1 The effect of the artificial bicistronic operon and the use of *sinIR* transcriptional terminator as a 3'-stabilizing element

To investigate the role of the transcriptional terminator *sinIR* in regulation of gene expression in *B. subtilis*, the vector pKL04 was constructed with the *sinIR* terminator sequence fused between the *bgaB* and *gfp* genes. The hypothesis was that the presence of a transcriptional terminator functioning as a positive regulator would allow higher expression of the upstream gene *bgaB* and decrease of the activity of the downstream gene *gfp*.

The plasmids pKL02, pKL03 and pKL04 were transformed in *E. coli* strain DH10B and plated on LB plates containing 100 µg/ml of ampicillin in the absence of IPTG. As shown in Fig. 9, analysis of the GFP fluorescence indicates that the plasmid pKL03 (no terminator present) exhibits high GFP fluorescence as compared to the plasmid pKL02 (no *gfp*) and the terminator-test vector pKL04. This result shows that the P_{grac} promoter is leaky, allowing mRNA production, although at a low level in the absence of induction.

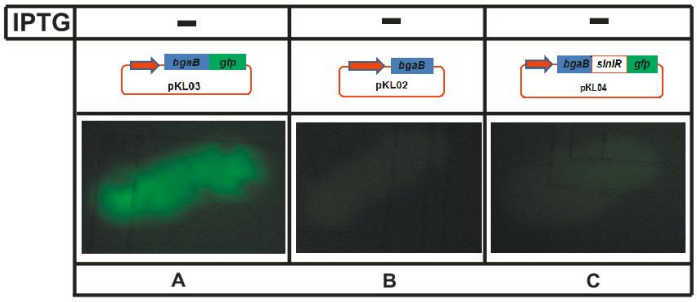

Figure 9: GFP fluorescence analysis of the bicistronic operon and the terminator-test vector transformed in *E. coli* in the absence of IPTG
(**A**) pKL03 as a positive control – no terminator ; (**B**) pKL02 as a negative control; (**C**) pKL04 containing the *sinIR* transcription terminator.

Next, these plasmids were transformed into *B. subtilis* strain 1012. It was expected to observe in this organism the same pattern shown above for *E. coli* strains. However, the strain 1012/pKL03 did not exhibit fluorescent cells either in the absence (Fig.10 - row 1) or in the presence of IPTG (Fig.10 - row 2).

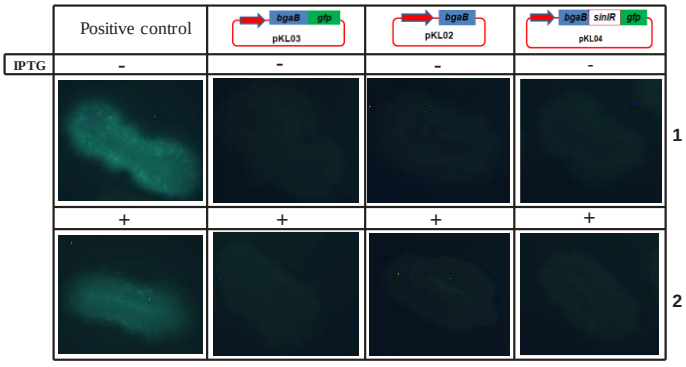

Figure 10: GFP fluorescence analysis of the bicistronic operon and the terminator-test vector transformed in *B. subtilis*
(**1**) positive control, pKL03, pKL02 and pKL04 non-induced with ITPG and (**2**) induced with 1 mM IPTG.

In order to investigate this different pattern of pKL03 in *E. coli* and in *B. subtilis*, the strain DH10B/pKL03 was also plated in the presence of IPTG. Interestingly, only a portion of cells showed fluorescence (Fig. 11). This result indicates that the construction of the plasmid

containing the bicistronic operon does not present a full structural stability. To test this hypothesis, portions of the fluorescent and non-fluorescent cells of the *E. coli* strain DH10B/pKL03 were plated in the absence and presence of IPTG (Fig. 12).

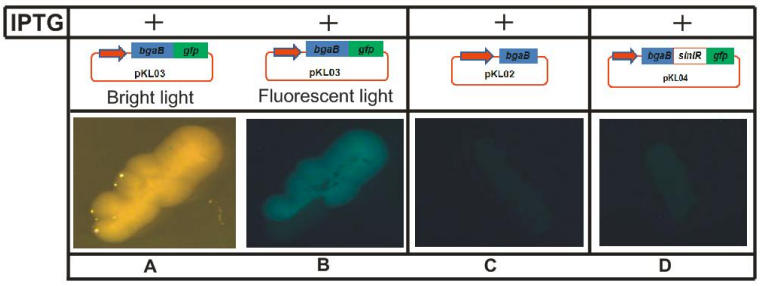

Figure 11: GFP fluorescence analysis of the bicistronic operon and the terminator-test vector transformed in *E. coli* in the presence of IPTG
(A and **B**) pKL03 – no terminator; (**C**) pKL02 as a negative control; (D) pKL04 containing the *sinIR* transcription terminator. IPTG concentration: 1 mM

3.1.1 The GFP fluorescence analysis in the artificial bicistronic operon

Four random single colonies of *E. coli* strain DH10B/pKL03 named here pKL03A, B, C and D, were plated in LB plates containing 100 µg/ml of ampicillin in the absence of IPTG. These colonies showed the same pattern observed on the Fig. 11, i.e., a mosaic of fluorescent and non - fluorescent cells (Fig. 12 A – row 1). Fluorescent cells were then plated in the absence of IPTG and the presence of fluorescent and non-fluorescent cells were again observed for all four colonies tested (Fig 12 A – row 3), corroborating the suggestion that this construction leads to instability of the plasmid. Moreover, upon induction with 1 mM IPTG the cells seemed to lose the ability to produce GFP, since almost no fluorescence was detected (Fig 12 A – row 5).

The analysis of the non-fluorescent cells in the absence of the inducer presented the same pattern observed for the fluorescent cells. Furthermore, independent of the presence or absence of IPTG, some colonies presented reduced growth and GFP – minus phenotype (Fig. 12 B – rows 2 and 4). When the non-fluorescent cells were plated in the presence of IPTG, no fluorescence was recovered (Fig 12 B – row 5).

In order to verify whether this mosaic pattern observed for GFP fluorescence is due to plasmid instability and not to mutations present in the *gfp* gene, the plasmids pKL03A, B C and D were sequenced and no mutations were localized either within the gene or within the ~ 100 bp flanking regions (data not shown).

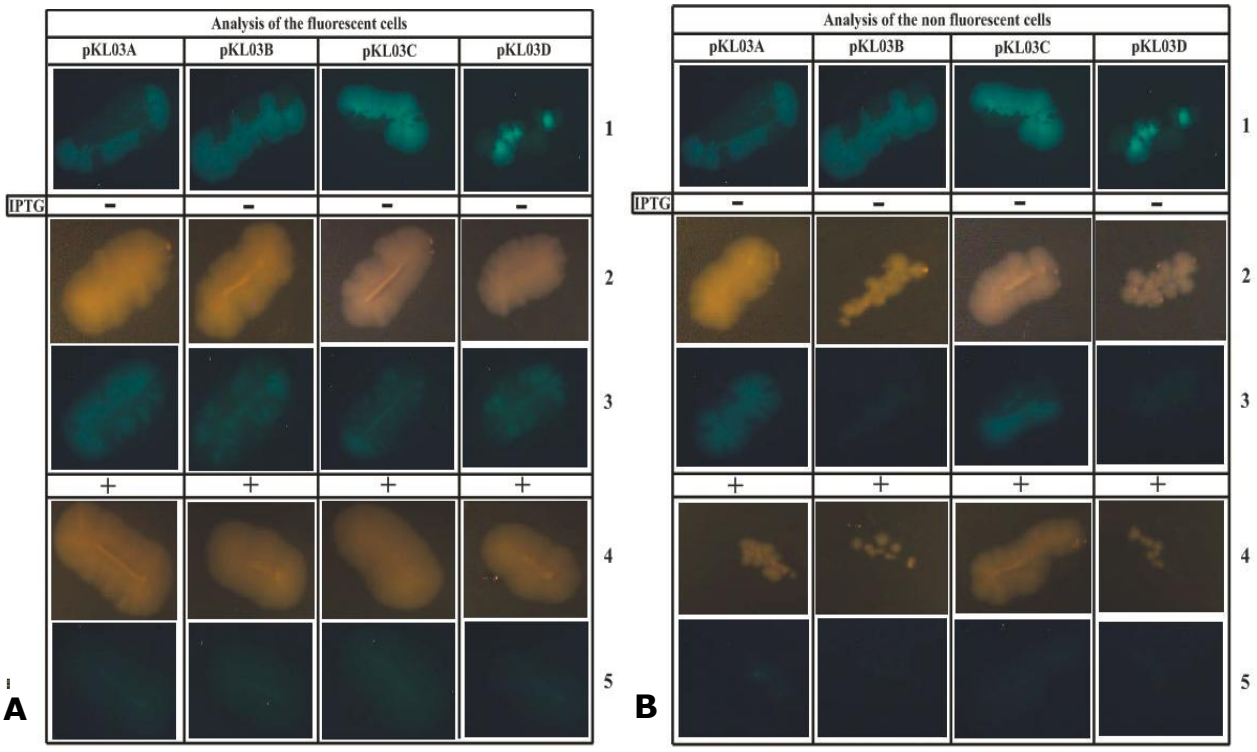

Figure 12: GFP analysis of the fluorescent (A) and non – fluorescent (B) cells of four random colonies of *E. coli* strain DH10B/pKL03
1 – from left to right: pKL03A, B, C and D in the absence of IPTG. **2** and **4:** bright light images. **3** and **5:** fluorescent light images in the absence and presence of IPTG, respectively.

3.1.2 **The BgaB activity analysis in the artificial bicistronic operon**

Next, I analyzed the *bgaB* expression in these four colonies tested. When fluorescent cells were grown in the absence of IPTG, the mixture of blue and white cells was observed (akin the mixture of fluorescent and non-fluorescent cells) (Fig. 13 A – row 2); and upon induction with 1 mM IPTG, these colonies showed lower *bgaB* expression (Fig. 13 A – row 3). The analysis of the non-fluorescent cells showed again a reduced growth phenotype in the absence and presence of

inducer (Fig. 13 B – rows 2 and 3) and also a lower *bgaB* expression compared with cells not induced (Fig. 13 B – row 2).

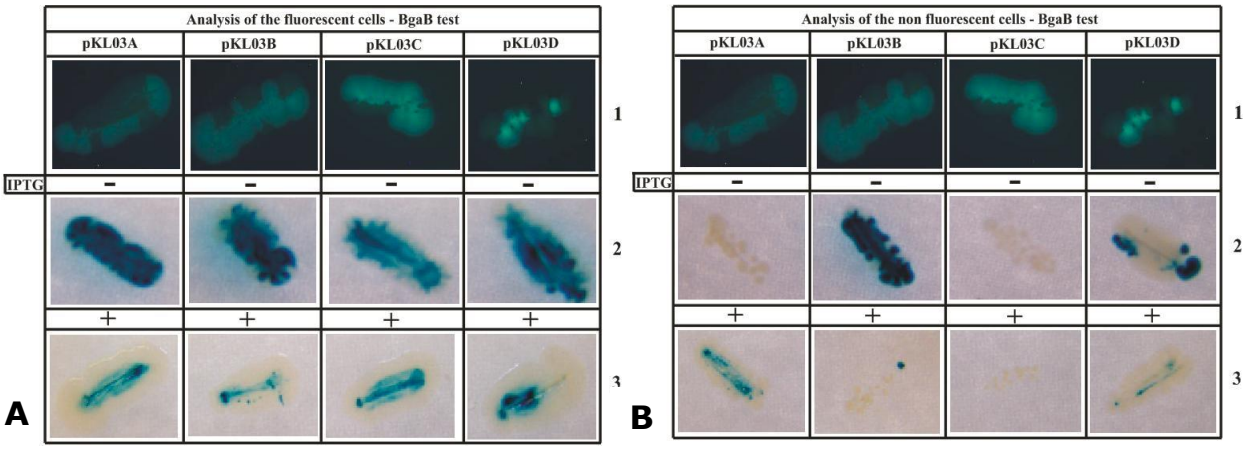

Figure 13: BgaB analysis of the fluorescent (A) and non–fluorescent (B) cells of four random colonies of *E. coli* strain DH10B/pKL03
1 – from left to right: pKL03A, B, C and D in the absence of IPTG; and **2** and **3:** in the absence and presence of IPTG, respectively.

After the analysis of BgaB in *E. coli*, I investigated the synthesis of this protein in *B. subtilis* cells. Different from what was observed for the *gfp* expression, the *bgaB* expression did not show a mosaic pattern. Plated on indicator plates containing XGal, the *B. subtilis* strain 1012/pKL03 grew normally and did not present a mixture of blue and white cells either in the absence or presence of IPTG (Fig 14).

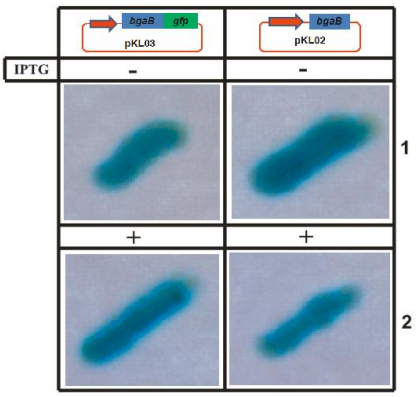

Figure 14: BgaB analysis of the *B. subtilis* strains 1012/pKL03 and 1012/pKL02
(**1** – from left to right): pKL03 and pKL02 (positive control) in the absence of IPTG and (**2**) in the absence of IPTG 1 mM.

Next, a quantitative experiment was performed to analyze the activity of BgaB in the construction of the bicistronic operon. For this purpose, measurement of the β-galactosidase activity was performed as described in 2.6.3. In brief, single blue colonies of the *B. subtilis* strains 1012/pKL02 (no *gfp*) and 1012/pKL03 were grown at 37°C in LB medium and induced with 1 mM IPTG. Samples were collected at t = 0 (before induction) and at t = 2, 4 and 6 h. The activity was determined as previously described (Mogk *et al.*, 1996). The experiment was performed three times and the mean value of the BgaB activity was given for each strain.

After 6 h the positive control 1012/pKL02 showed a considerably higher activity than that observed with strain 1012/pKL03, illustrated as blue and green columns, respectively (Fig. 15). This indicates that the presence of *gfp* exerts an effect on the stabilization and expression of the *bgaB* transcripts.

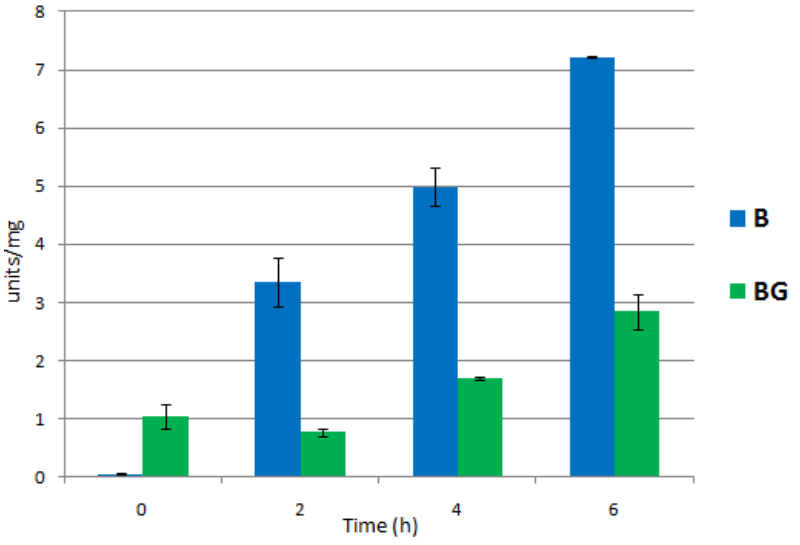

Figure 15: Quantitative measurement of the β-galactosidase activity of the bicistronic *B. subtilis* operon
pKL02 (**B** - blue column) as a positive control; pKL03 (**BG** – green column).

The last experiment was performed to verify whether the structural instability of the bicistronic operon-containing plasmid in *E. coli* was due to a rearrangement of the plasmid. Four restriction enzymes were used in different reactions to investigate whether those plasmids presented a different pattern of fragments (compared with the pattern expected based on the sequence of the plasmid) when treated with enzymes that cleave the flanking region of the bicistronic operon.

The band sizes detected on a 0.8 % agarose gel did not show any indication of rearrangements in the pKL03 (data not shown).

It was concluded that the construction of the bicistronic operon was not suitable for investigating the role of transcriptional terminators in *B. subtilis*. The plasmid pKL03 does show a structural instability regarding the expression of both *bgaB* and *gfp* in *E. coli*. Moreover, the construction of the bicistronic operon shows to result in a considerable lower BgaB activity and an unexpected lack of *gfp* expression in *B. subtilis*.

3.2 The expression of α-amylase in *B. subtilis* by pKL01

To analyze expression and secretion of α-amylase into the supernatant of *B. subtilis* cells, the plasmid pKL01 was constructed. It contains the *amyQ* gene under control of the IPTG-inducible P_{groES} promoter. The aim of this part of my thesis was to improve secretion of the reporter enzyme α-amylase by two different experimental approaches.

The first attempt to improve secretion of α-amylase into the supernatant was based on using different IPTG-concentrations, 0.1, 1 and 3 mM. To test the hypothesis that increasing the concentration of the inducer provides a higher level of secretion, three different experiments were carried out: a plate assay, a Northern and a Western blot. In these analyses, the *B. subtilis* strain 1012/pKL01 was induced with 0.1, 1 and 3 mM IPTG.

In the plate assay, single colonies grew for 12 h on indicator plates containing 1% soluble starch and were stained with I_2/KI. The α-amylase once located outside of the cell is able to degrade the starch present in the medium and this reaction yields the formation of a halo surrounding the colonies (Fig. 16).

As the plates containing 0.1, 1 and 3 mM IPTG showed approximately the same halo sizes (Fig. 16 B, C and D, respectively) we could conclude that the increase of IPTG concentration does not

influence secretion of α-amylase into the medium. A plate without IPTG was used as a control (Fig. 16 A) and clearly shows that the promoter present in the plasmid presents a considerably high basal level of activity, since the colonies show a clear halo around the cells demonstrating that the promoter is leaky as already published (Nguyen *et al.*, 2007).

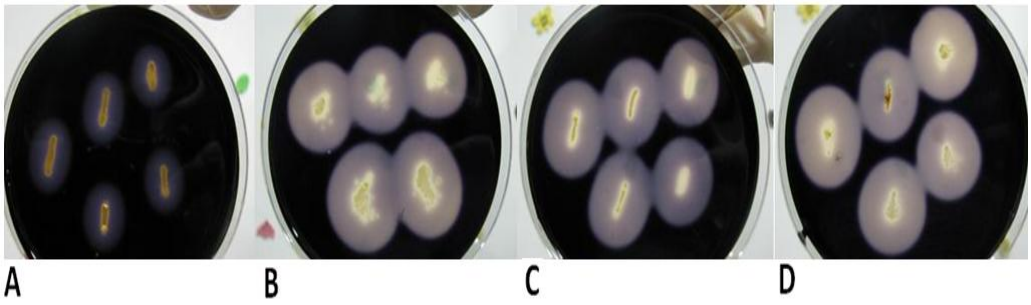

Figure 16: Visualization of extracellular α-amylase activity of the *B. subtilis* strain 1012/pKL01 induced with different concentrations of IPTG
Single colonies of 1012/pKL01 grown for 12 h on indicator plates containing 1% insoluble starch and stained with I_2/KI. **A**: control plate without IPTG; **B**, **C** and **D**: plates containing 0.1, 1 and 3 mM IPTG, respectively.

Three hypotheses arose based on this first result:

1. The increase of inducer does not interfere with secretion of the α-amylase;

2. The highest concentrations of inducer lead to a high level of protein synthesis inside of the cells, guiding these proteins to form aggregates known as inclusion bodies and, therefore, allowing only a partial secretion of the total amount of protein into the supernatant (Villaverde and Carrio, 2003; Fahnert *et al.*, 2004; Ventura and Villaverde, 2006);

3. If the cells are driven to overproduce secretory proteins, these large amounts of proteins are targeted to the SEC translocon in the bacterial membrane and caused it to become jammed. This jamming process avoids the complete and correct function of the translocation machinery, attaching these preproteins to the membrane and consequently not allowing their secretion (Campo *et al.*, 2004; Breukink, 2009);

4. It is also possible that there is a mixture of 2 and 3. A high level of protein synthesis might lead to the formation of protein aggregates and also jam the bacterial membrane.

To test whether the increase of inducer does interfere with transcription of *amyQ*, a Northern blot experiment was performed to measure the amount of mRNA synthesized. Total RNA of the strain 1012/pKL01 was isolated from cultures grown in LB medium in the presence of 0.1, 1 and 3 mM IPTG. As deduced from Fig. 17, the increase of the inducer IPTG is followed by a concomitant increase in the amount of *amyQ* transcripts. Therefore, IPTG does not interfere with transcription of *amyQ*.

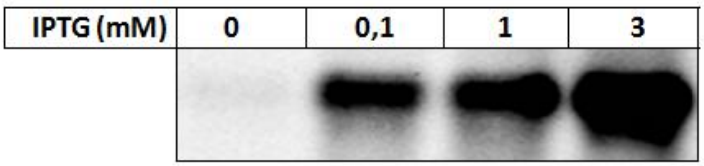

Figure 17: Northern blot analysis for quantification of the *amyQ* gene in the *B. subtilis* strain 1012/pKL01
The strain 1012/pKL01 was grown in LB medium and total RNA was analyzed using *amyQ* antisense RNA. From left to right: control without IPTG; samples induced with 0.1, 1 and 3 mM IPTG, respectively. Concentration applied in each lane: 10 µg/µl

Next, I checked for the presence of α-amylase present within the cytoplasm and attached to the membrane. A large amount of proteins synthesized within the cells can lead to protein insoluble aggregates in the cytoplasm, which impair the translocation of secretory proteins into the supernatant. To emphasize this assumption, a Western blot experiment was carried out.

In order to verify whether a certain amount of protein is in fact retained inside the cells in strain 1012/pKL01, cells were induced with 0.1, 1 and 3 mM IPTG, and subsequently fractioned into cytoplasmic (soluble), membrane (insoluble) and supernatant fractions (Fig. 18). Since the production of α-amylase in *B. subtilis* occurs during both the exponential and stationary phases of growth (Leloup *et al.*, 1997), samples were collected at the beginning of the stationary phase.

Even though there is more α-amylase in the supernatant as compared to the amount retained in the two other fractions, there is still a considerable amount of α-amylase that accumulated in the cytoplasm and at the cytoplasmic membrane. It can be concluded that either the α-amylase present in the cytoplasm and attached to the membrane is still on the way to be secreted or is inactivated due to aggregation.

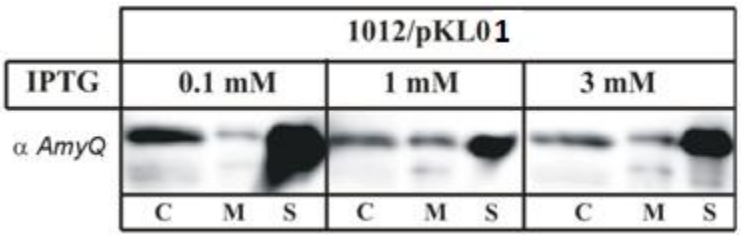

Figure 18: Western blot analysis for quantification of α-amylase in the *B. subtilis* strain 1012/pKL01
The strain 1012/pKL01 was grown in LB medium and the samples were induced with 0.1, 1 and 3 mM (from left to right) at an OD_{578} of 0.8 and collected when reached the beginning of stationary growth phase. **C:** cytoplasmic fraction; **M:** membrane fraction; and **S:** supernatant fraction. Antibodies raised against AmyQ was used. Protein concentration applied in each lane: 10 µg/µl.

These results led us to test the fourth hypothesis. I carried out two different experiments to find out whether the overproduced and accumulated proteins inside of the cells avoid translocation into the supernatant by blocking the translocation machinery. The first one was performed to verify whether overexpression of *B. subtilis secA* can increase secretion of α-amylase. The second experiment was to construct an artificial *secYEG* operon in order to enhance the amount of genes coding for the translocon components to facilitate the translocation of the remaining α-amylase inside the cells.

3.2.1 **Overexpression of *B. subtilis secA* does not improve secretion of α-amylase**

To analyze whether overexpression of *secA* can increase secretion of α-amylase in the *B. subtilis* strain 1012/pKL01, the vector pWKML01 was used. This vector bears the *secA* wild-type gene of *B. subtilis* under the control of a xylose-inducible promoter. Its transformation into

1012/pKL01 resulted in the strain KL01. The two plasmids pWKML01 and pKL01 present within KL01 are compatible with each other.

As described before, the cytoplasmic and membrane protein SecA is an ATP-dependent motor protein and a central component of the Sec pathway. Thus, this experiment was carried out based on the hypothesis that an overproduction of SecA would improve the translocation machinery by raising the number of SecA molecules.

3.2.1.1 Overexpression of *secA*

The first step was to verify whether SecA was overproduced by pWKML01 in strain 1012/KL01. After 12 h of induction with either 0.25 or 0.5 % xylose, samples were collected and the cytoplasmic fraction was analyzed. An overproduction of SecA was observed when the strain KL01 was induced with 0.25 as well as with 0.50 % xylose at 12 and 24 h as compared with the non-induced control (Fig. 19). Nonetheless, the samples collected at 24 h showed a reduced amount of SecA, indicating a considerable degradation of the protein after this stage. Based on this result, we could conclude that 0.25 % is the suitable concentration of xylose to induce overproduction of SecA by pWKML01.

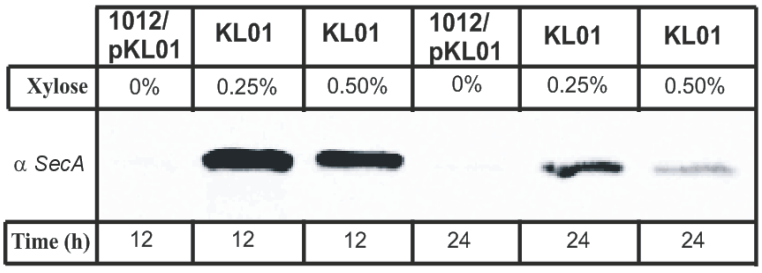

Figure 19: Western blot analysis for quantification of SecA in the *B. subtilis* strain KL01
The cells were grown in LB medium and were induced at an OD_{578} of 0.8 by addition of xylose. Samples were collected at 12 (columns on the left side) and 24 h (columns on the right side). The strain 1012/pKL01 was used as a negative control. Antibodies raised against SecA were used. Protein concentration applied in each lane: 10 µg/µl

3.2.1.2 Co-expression of *secA* and *amyQ*

Next, the effects of coproduction of SecA and α-amylase secretion in the strain KL01 was analyzed in samples induced with 0.25% xylose and 0.1 mM IPTG, and collected after 12 h of incubation. For this purpose, the analyses of overproduction and secretion of α-amylase were performed in fractioned samples, separated into cytoplasmic, membrane and supernatant fractions. The strain 1012/pKL01 was used as a negative control.

As shown in the Fig. 20, we observed that strain KL01 retains approximately five and threefold less α-amylase in the cytoplasm and in the cytoplasmatic membrane, respectively, in comparison with the amount present in the same compartments in the control strain. However, the contrary was observed when the supernatant fraction was analyzed. In the strain KL01, around half of the total amount of α-amylase secreted by strain 1012/pKL01 was detected. This result indicates that coproduction of SecA interferes with the total amount of α-amylase synthesized in the cells.

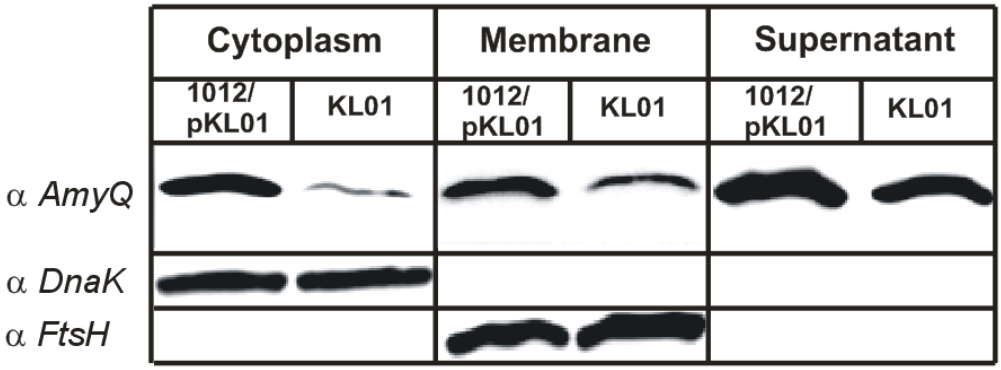

Figure 20: Western blot analysis for quantification of α-amylase in the *B. subtilis* strains 1012/pKL01 and KL01 (pKL01 + pWKML01)
Both strains were grown in LB medium and were induced with 0.1 mM IPTG (1012/pKL01) plus 0.25 % xylose (KL01) at an OD_{578} of 0,8. Samples were collected at 12 h. From left to right: cytoplasmatic, membrane and culture supernatant fractions. Antibodies raised against DnaK and FtsH were used as cytoplasmatic and membrane loading controls, respectively. Concentration applied in each lane: 10 µg/µl.

This experiment was carried out three times and also at 30°C (data not shown) and showed always the same pattern as observed above. To complement this data, a measurement of α-amylase activity in the culture supernatant was performed. As presented below, the control strain showed slightly higher activity compared with the strain KL01 (Fig. 21). This test corroborated the results obtained with by the Western blot experiment, i.e., the strain coding for overproduction of SecA secretes less protein than the strain which lacks the plasmid pWKML01.

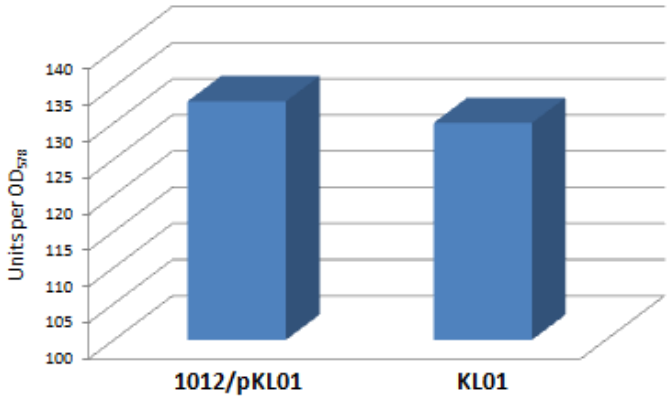

Figure 21: Measurement of α-amylase activity in the *B. subtilis* strains 1012/pKL01 and KL01
Both strains were grown in LB medium and were induced with 0.1 mM IPTG (1012/pKL01) plus 0.25% xylose (KL01) at an OD_{578} of 0.8. Samples were collected at 12 h.

3.2.2 The artificial *secYEG* operon increases the amount of secreted α-amylase in *B. subtilis*

The process of protein translocation in bacteria is performed throughout a heterotrimeric protein complex designated as SecYEG. This complex forms a pore in the cytoplasmatic membrane acting as a protein-conducting channel. The second experiment performed to achieve overproduction and secretion of α-amylase in *B. subtilis* cells was based on the construction of an artificial *secYEG*. I questioned "Does a higher expression of these genes can enhance the amount of translocons in the cytoplasmic membrane and therefore increase the secretion of α-amylase into the supernatant?"

The artificial operon was constructed with the *B. subtilis secY*, *secE* and *secG* genes. The plasmids pKL10 and pKL12, where the artificial operon is under the control of the IPTG-inducible P_{spac} promoter and the xylose-inducible P_{xylA} promoter, respectively, were used.
After construction of both plasmids, two experiments were performed:

(i) The *amyQ* gene, under the control of the P_{xylA} promoter (pKL11), was integrated into the *B. subtilis* strain IHA01 (1012 *lacA::spec*) at the *lacA* locus resulting in strain KL02. Then, the plasmid pKL10 was introduced into that strain. This experiment was performed to verify whether a high copy number plasmid can overexpress *secY*, *secE* and *secG* and enhance secretion of α-amylase in a strain containing the *amyQ* gene integrated into the chromosome.

(ii) The *secYEG* operon under the control of the P_{xylA} promoter was integrated into the *B. subtilis* strain IHA01, resulting in the strain KL03. Then, the plasmid pKL01 was added. In this experiment the opposite approach was taken, i. e., I verified whether α-amylase secretion can be increased being produced by a high copy number plasmid in a strain overexpressing *secY*, *secE* and *secG* when these genes are integrated into the chromosome.

Considering that there is a lower expression rate for integrative plasmids in comparison to replicating plasmids, I first verified the production of α-amylase by the strain KL02/pKL10. The strain was induced with 0.25 % xylose plus 0.1 mM IPTG and the culture supernatant was collected after 12 h. The strain KL02 was used as a negative control. A low concentration of protein secreted into the supernatant was detected by Western Blot (data not shown) and I concluded that there was no difference between the amount of α-amylase secreted by the strains KL02/pKL10 and KL02. Therefore, all the following results were obtained performing the second experiment.

3.2.2.1 Overexpression of *secY, secE* and *secG*

As described above for the SecA analyses, the first step was to verify whether the proteins SecY, SecE and SecG were overexpressed by the strain KL03. A Western blot experiment was performed to investigate expression of *secY*. The strain IHA01 (no *secYEG* operon integrated into the chromosome) was used as a control and the total cell lysate of both strains was analyzed. Based on the result presented on Fig. 22, we concluded that the induction of *secY* gene expression in the strain KL03 led to an approximately threefold overproduction of SecY.

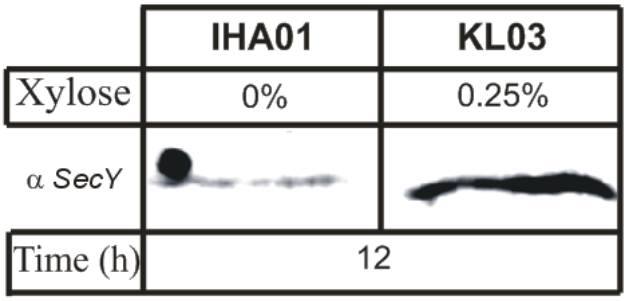

Figure 22: Western blot analysis for quantification of SecY in the *B. subtilis* strain KL03
Strain 1012/pKL01 was grown in LB medium and the samples were induced with 0.25% xylose at an OD_{578} of 0.8 and collected at 12 h. Antibodies raised against SecY were used. Protein concentration applied in each lane: 10 µg/µl

Since there were no antibodies available for SecE and SecG, expression of the genes *secE* and *secG* was evaluated by Northern blot. The strain KL03 was induced with 0.25 % xylose (set as t=0) and collected at an OD_{578} of 1.5, 2.5 and 3.5 (set as t=1, 2 and 3, respectively). Total RNA was isolated and subjected to Northern blotting, which was probed with two different antisense RNAs, one complementary to the *secEG* transcript, comprising a full transcription of both genes (465 bp); and other to the *dnaK* transcripts, used as a loading control. When antisense RNA complementary to the *secEG* was used to probe the Northern blot, a transcript with a length of the *secEG* was predominating, without the presence of additional bands. Furthermore, I was able to detect a progressive increase of transcripts from t=0 to t=3, indicating that the *secE* and *secG* genes were expressed under the control of the xylose-inducible promoter in the strain KL03 (Fig. 23). In contrast, the amount of *dnaK* transcript remained unchanged as to be expected.

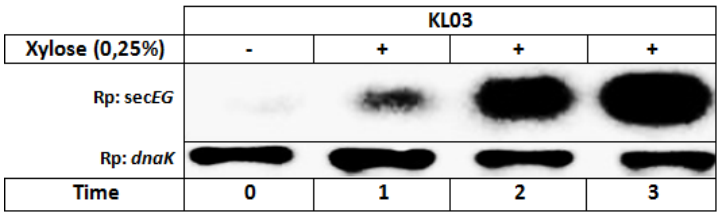

Figure 23: Northern blot analysis for quantification of the *secEG* gene in the B. subtilis strain KL03
The strain were grown in LB medium and the total RNA was analyzed using *secEG* antisense RNA. *dnaK* antisense RNA used as loading control. Rp: riboprobe. Concentration applied in each lane: 25 µg/µl

3.2.2.2 Overexpression of α-amylase by the strain KL03

Next, the production and over secretion of α-amylase by the plasmid pKL01 was analyzed in the *B. subtilis* strain KL03. After 12 h of induction with 0.25 % xylose plus 0.1 mM IPTG, cells of strain KL03/pKL01 were collected and again fractioned into cytoplasmic, membrane and supernatant fractions. The strain IHA01/pKL01 was induced 0.1 mM IPTG and used as a control (Fig. 24).

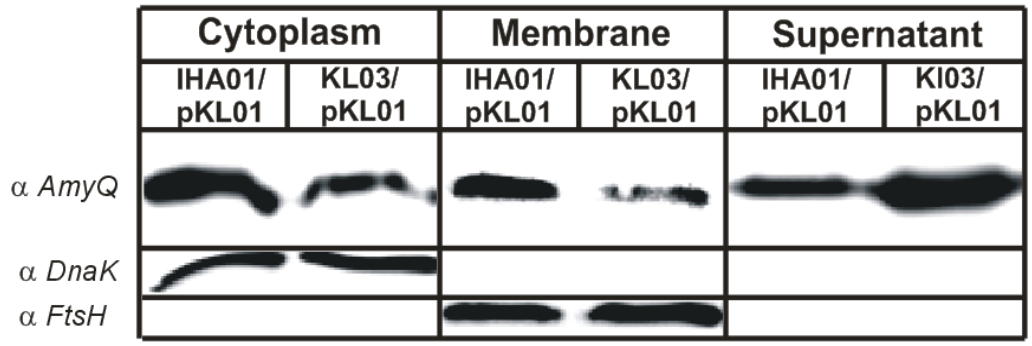

Figure 24: Western blot analysis for quantification of α-amylase in the *B. subtilis* strains IHA01/pKL01 and KL03/pKL01
Both strains were grown in LB medium and were induced with 0.1 mM IPTG (IHA01/pKL01 - (no *secYEG* operon integrated) plus 0.25 % xylose (KL03/pKL01 - *secYEG* operon integrated into the chromosome) at an OD_{578} of 0.8. Samples were collected at 12 h. From left to right: cytoplasmatic, membrane and culture supernatant fractions. Antibodies raised against DnaK and FtsH were used as cytoplasmatic and membrane loading controls, respectively. Protein concentration applied in each lane: 10 µg/µl.

The strain KL03/pKL01 showed a considerably smaller amount of protein in both the cytoplasmic and membrane fraction in comparison to the amount of protein present in the control strain, indicating that the increase in the proteins SecY, SecE and SecG contributes to the release of retained α-amylase inside of the cells. Furthermore, in contrast to what was observed by the coproduction of SecA, the coproduction of *secYEG* led to an approximately threefold higher amount of α-amylase secreted into the culture supernatant.

In order to confirm that the strain KL03/pKL01 secretes a higher amount of protein into the supernatant, a measurement of the α-amylase activity was performed. As shown in Fig. 25, this strain showed higher α-amylase activity as compared with the control strain. This result confirms the data obtained with the Western blot experiment and therefore it was concluded that the increase of the proteins SecY, SecE and SecG encoded by the *secYEG* artificial operon does enhance the secretion of an active α-amylase into the culture supernatant of *B. subtilis* cells.

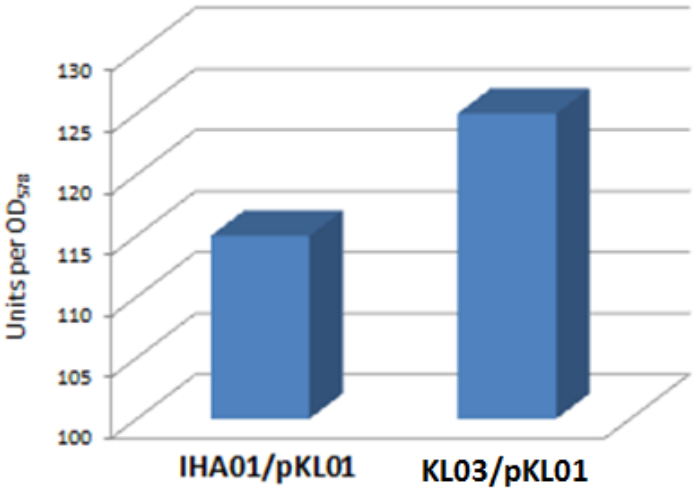

Figure 25: Measurement of α-amylase activity in the *B. subtilis* strains IHA01/pKL01 and KL03/pKL01
Both strains were grown in LB medium and induced with 0.1 mM IPTG (IHA01/pKL01) and with 0.25% xylose plus 0.1 mM IPTG (KL03) at an OD_{578} of 0.8 and collected at 12 h

3.3 Transposon mutagenesis in *B. subtilis*

3.3.1 Detection of mutants able to increase secretion of α-amylase

Can transposon mutagenesis inactivate genes that might improve secretion of proteins in *B. subtilis*? In order to answer this question, the transposon mutagenesis strategy was used to inactivate gene(s) whose product might regulate directly or indirectly the secretion of α-amylase.

In the first experiment, the pMarA plasmid was used (Le Breton *et al*., 2006). It is a transposon–delivery plasmid containing the *mariner*-based transposon TnYLB-1 and a *mariner-Himar*1 transposase gene under the control of a σ^A-dependent promoter.

The pMarA was transformed into *B. subtilis* strain 1012 and plated on LB containing Kan (resistance gene present in the transposable element) at 30 °C. Colonies were tested for plasmid-associated properties as described in 2.8.1. The strain 1012/pMarA was used as a receptor strain and the plasmid pKL01 was then transformed, generating the strain KL12 (*B. subtilis* strain 1012 containing both pMarA and pKL01). Transposon mutagenesis was carried out in LB medium at 50 °C and appropriate dilutions were plated on LB containing Kan plus Cm and on LB containing Erm and incubated at 37 °C. Then, the measurement of viable bacteria, calculated as cfu/ml (colony-forming units per milliliter) was performed. Approximately 97% of the thermo resistant Kan^r plus Cm^r clones (cfu/ml = $4x10^7$) were sensitive to Erm (cfu/ml = $1x10^6$), the antibiotic resistance encoded by the plasmid but not included within the transposable element. This result indicates that only ~ 3% of the clones after transposition events did not loose the plasmid, and therefore, the transposon TnYLB-1 was not inserted into the chromosome. These percentages were obtained by Erm^r/Kan^r representing the colonies that displayed the plasmid-encoded antibiotic resistance (Erm^r) versus the transposon-encoded resistance (Kan^r).

Roughly, 10.000 single colonies were screened for halo sizes after transposon mutagenesis. These colonies were plated on LB agar containing Kan plus Cm and 1% soluble starch. It was expected that the transposition events could inactivate genes involved in secretion of α-amylase. These mutants could be detected in the indicator plates by showing bigger halos surrounding the colonies. All the colonies screened presented approximately the same halo size.

3.3.2 **A modified transposon is able to induce gene expression**

In the second experiment concerning the use of the transposon mutagenesis strategy, I devised a modified transposon containing a xylose-expression cassette in order to induce expression of gene products that might enhance secretion of α-amylase. The plasmid pMarA was used as a

backbone vector and the cloning of the xylose-expression cassette generated the modified transposon named pKL13, as described in 2.8.2.

In order to demonstrate that this modified transposon is still able to transpose, the plasmid pKL13 was transformed into *B. subtilis* strain 1012 and transformant colonies were selected and screened for plasmid-associated properties as described above. Transposon mutagenesis was carried out and appropriate dilutions were plated on LB containing Kan and LB containing Erm plates. The measurement of viable bacteria indicated that approximately 20% of the Kan^r clones (cfu/ml = $8x10^6$) were resistant to Erm (cfu/ml = $1.5x10^6$), showing that in about 80% of the clones showed transposition events where the plasmid pKL13 was cured and the transposon inserted into the chromosomal DNA.

The second step was to verify whether these transposition events might allow or improve expression of genes due to the presence of the xylose-inducible promoter. Based on the principle that transposons are inserted randomly into the chromosomal DNA, this transposable element can possibly trigger expression of an inactivate gene. To test this hypothesis, the promoter-less chloramphenicol resistance marker (*cat* gene) was used as a reporter gene and integrated into the *B. subtilis* chromosomal DNA.

Concerning the presence of secondary structures that might impair the expression of genes, the *cat* gene was inserted in both possible orientations at two different loci. In brief, the plasmids pKL14 and pKL16 containing the *cat* gene in one orientation were integrated into the *amyE* and *thrC* loci generating the strains KL04 and KL06 (*amyE*::*i-cat* and *thrC*::*i-cat*), respectively. The plasmids pKL15 and pKL17 carrying the *cat* gene in the other orientation were integrated also into the *amyE* and *thrC* loci generating the strains KL05 and KL07 (*amyE*::*cat* and *thrC*::*cat*), respectively.

To confirm that there was no expression of the *cat* gene in the constructed strains, single colonies from each strain were plated on LB plates containing Cm at 2.5, 5 and 10 µg/ml plus 1 % xylose. This test was performed in the presence of antibiotics and inducer to guarantee that the

expression of the *cat* gene was only possible if induced by the xylose-inducible promoter after carrying out the transposon mutagenesis experiment. The only strain that could form colonies at the concentration of Cm at 2.5 and 5 µg/ml was the strain KL07. The other strains showed no growth, independent of the antibiotic concentration. This result led to the following conclusions: i) The strain KL07 is only sensible to a high concentration of antibiotic such as 10 µg/ml. Thus, the colonies which grew at low concentrations of chloramphenicol might reflect subpopulations of cells that had become resistant to the presence of Cm at 2.5 and 5 µg/ml; or ii) the *cat* gene present in this strain is transcribed in the presence of low concentrations by the promoter of the *thrC* locus.

Consequently, further experiments were carried out using strains KL04, KL05 and KL06. I first tested the transposition frequency of the plasmid pKL13 in the *B. subtilis cat*-containing strains. Here, the plasmid pKL13 was transformed and transposon mutagenesis was carried out as described above. Kan^r clones, representing transposition events, appeared with approximately equal frequencies (~10^{-2}) regardless of the strain used. Furthermore, more than 90% of the thermo resistant Kan^r clones were sensitive to Erm (Table 7).

Table 7: pKL13 transposition in the *cat*-containing strains
Transposition frequency was calculated as Kan^r colonies/LB colonies. Erm^r/Kan^r represents the percentage of colonies that displayed the plasmid-encoded antibiotic resistance (Erm^r) versus the transposon-encoded resistance (Kan^r).

	Viable cell count (cfu/ml)				
Strain	**Kan^r 5 µl/ml**	**Erm^r 1µl/ml**	**LB**	**Transposition frequency**	**Erm^r/Kan^r**
KL04/pKL13 (*amyE::cat*)	1.5 x 10^7	2 x 10^5	4.6 x 10^7	3x10^{-1}	1.3%
KL05/pKL13 (*amyE::i-cat*)	5.5 x 10^6	1 x 10^4	9.7 x 10^6	5.6x10^{-2}	1.8%
KL07/pKL13 (*thrC::i-cat*)	7 x 10^5	5 x 10^4	7 x 10^7	9.3x10^{-2}	7.1%

Second, to check whether it was possible to induce expression of the *cat* gene transposon mutagenesis was carried out with additional steps. After ~5 h at 50 °C, the cultures KL04/pKL13, KL05/pKL13 and KL07/pKL13 were used to inoculate a new LB culture in the presence of Cm at 10 µg/ml plus 1% xylose. Subsequently, the culture was grown overnight at 37 °C and diluted aliquots were plated on LB containing Cm 10 µg/ml plus 1 % xylose and on LB without xylose.

Chloramphenicol-resistant colonies of the three *cat*-containing strains grew on the selective plates, indicating that the *cat* gene was expressed by the xylose-inducible promoter present in the transposon. In order to test this assumption, 50 colonies of each strain were replated on LB plates containing 5 µg/ml Kan or 1 µg/ml Erm and all the colonies were kanamycin-resistant and erythromycin-sensitive, as expected. Moreover, less than 1% of the colonies of strains KL04/pKL13 and KL05/pKL13 and only ~ 10 % of the colonies of the strain KL07/pKL13 grew on LB in the absence of inducer (Table 8), indicating that the majority of the chloramphenicol resistant colonies derived from the transposon mutagenesis is dependent of the presence of xylose. This result confirms that the promoter-less *cat* gene can be expressed upon induction of the modified transposable element inserted into the chromosomal DNA.

Table 8: Chloramphenical resistant colonies in the presence and absence of xylose
Cm^r/Cm^r + xylose represent the percentage of colonies that were grown in the absence versus colonies that were grown in the presence of 1 % xylose.

	Viable cell count (cfu/ml)		
Strain	**Cm^r + xylose**	**Cm^r** (5 µl/ml)	**Cm^r / Cm^r + xylose**
KL04/pKL13 (*amyE::cat*)	9.7×10^9	4.5×10^5	0.004%
KL05/pKL13 (*amyE::i-cat*)	9.8×10^9	8×10^6	0.08%
KL07/pKL13 (*thrC::i-cat*)	9.1×10^6	9.8×10^5	10.7%

3.3.3 **Analysis of the transposon insertion sites**

To identify the exact sequences surrounding the transposon integration sites and to test whether the insertions were at random, chromosomal DNA of four Cm^r clones of each strain was isolated

and PCR with primers comprising sequences within the beginning of the *cat* gene and the end region of the xylose-inducible promoter (present within the transposon TnYLB-1) was performed (Fig. 27 A). If the transposon had inserted randomly into the chromosome in these clones, different sizes of bands would be expected. Four out of 12 colonies showed successful amplification yielding three different patterns of bands (Fig. 26, lanes 2, 4 and 5). Interestingly, these clones were all derived from Cm^r clones of the strain KL04/pKL13. These four new mutant strains were names KL08, KL09, KL10 and KL11.

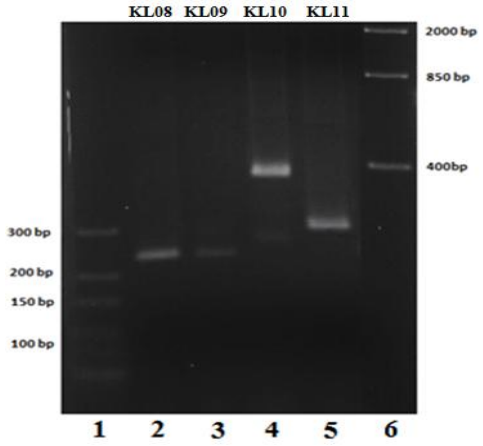

Figure 26: PCR analysis of TnYLB-1 plus P_{xylA} promoter insertion in the *B. subtilis* strain KL04
PCR product of four Cm^r clones isolated at 37° C from KL04/pKL13. Lanes 2 to 5: the strains KL08 to KL11, respectively. Lanes 1 and 6: DNA ladder showing fragment sizes in bp.

The PCR products were then sequenced and analyzed for conserved nucleotides using the WebLogo program (www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi) (182). The four transpositions that were examined resulted in three different locations on the chromosome (Fig. 27 B). The strains KL08 and KL09 presenting the same band sizes showed that the transposon was inserted exactly at the same position, 71 bp upstream the Shine-Dalgarno sequence of the *cat* gene. In the sequence analysis of the strains KL10 and KL11 we could identify that the transposon had inserted 274 and 133 bp upstream the Shine-Dalgarno, respectively, as shown by the band pattern observed in the Fig. 26.

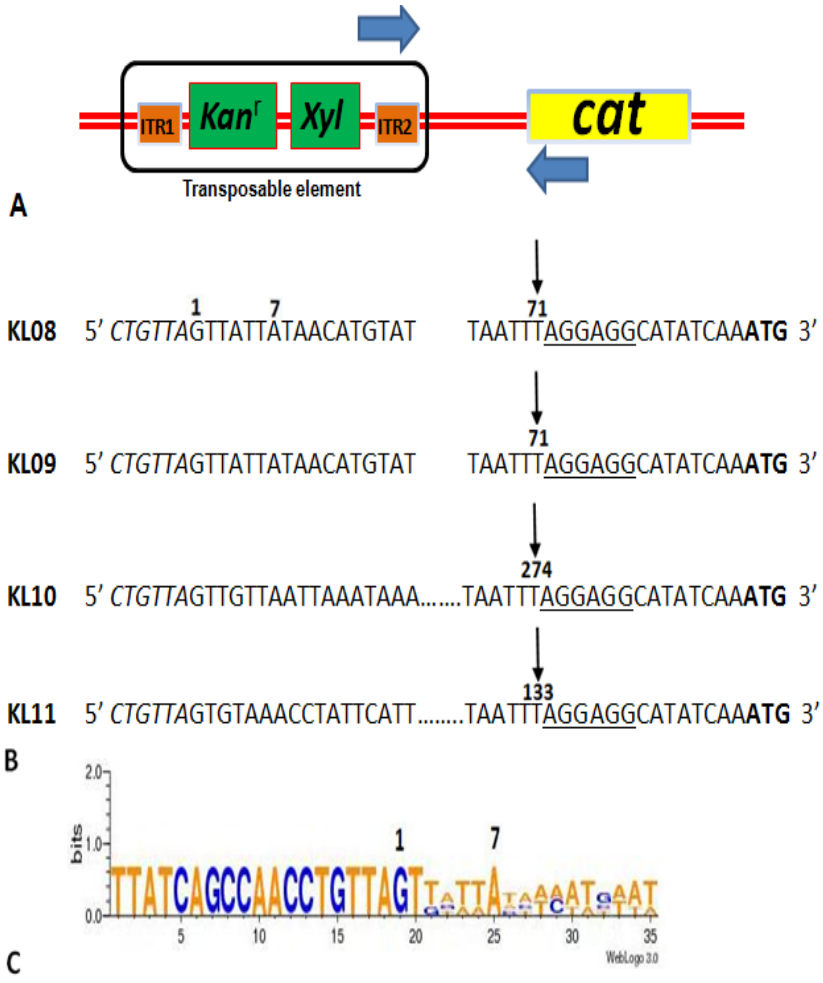

Figure 27: Sequence surrounding the transposon insertion sites
(**A**) Localization of the primers (blue arrows) used to amplify the DNA chromosomal (red lines) sequences between the transposition insertion sites and the *cat* gene in the Cm^r clones. (**B**) Sequences of the four Cm^r clones (strains KL08 to KL11). The end of the transposable element is shown in italic. The Shine-Dalgarno sequence is underlined and the ATG start codon of the *cat* gene is shown in bold face. The nucleotides at positions 1 and 7 bases represent the "GT" dinucleotide target and the seventh base downstream of the transposon region, respectively. The distance (in bp) between the end region of the transposon and the Shine-Dalgarno sequence of the *cat* gene nucleotide is indicated by black arrows. (**C**) Consensus analysis of the transposon insertion site sequences displayed along the horizontal axis. The height and order of each letter correspond to the relative frequency of the nucleotides. Nucleotides used most frequently are on top.

The sequence data indicated that the transposon had inserted preferentially at "GT" dinucleotide sites. The alignment of the sequences also highlights a preference for "A" nucleotide seven bases downstream of the transposon insertion sites. Aside for this possible "A" nucleotide preference, there were no additional sequences conserved among the insertion sites (Fig. 27 C).

Here, we concluded that the transposon TnYLB-1 containing a P_{xylA} promoter is able to induce the expression of the promoter-less *cat* gene and, as expected, the transposon was inserted randomly into the chromosomal DNA.

4 Discussion

4.1 The use of the artificial bicistronic operon and its effect in both *E. coli* and *B. subtilis*

To date, only one 3' stabilizing element has been described in *B. subtilis*. Discovered in 1986, a 381 bp region containing the transcriptional terminator of the crystal protein (CrylAa) from *B. thuringiensis* was identified as a positive regulator that enhances the expression of a heterologous gene by providing protection from 3' to 5' exonucleases, and therefore stabilizing the RNA transcripts (Wong and Chang, 1986). A study performed two decades later addressed the importance of this transcriptional terminator for the stability of the *crylAa* gene itself in *B. subtilis* (Ramirez-Prado *et al.*, 2006). However, is has also been shown that transcripts containing the *crylAa* terminator might result in a poor efficiency of termination as well, indicating that the great stability attributed to this terminator depends on the construction that is performed (Hess and Graham, 1990).

Since there are few studies regarding 3'-stabilizing elements in *B. subtilis*, we selected one potential transcription terminator *sinIR*, to evaluate its influence on the decay of a stable transcript such as *bgaB*. The *sinIR* is a *B. subtilis* bicistronic operon coding for a DNA-binding protein and is involved in inhibition of sporulation and protease production (Gaur *et al.*, 1991; Shafikhani *et al.*, 2002). Analysis of this transcription terminator was performed using an artificial bicistronic operon containing the *bgaB* and *gfp* genes (pKL03). The goal here was to insert the *sinIR* sequence (pKL04) between both reporter genes based on the hypotheses that the presence of the terminator would stabilize the *bgaB* transcript and terminate transcription at its end, therefore, enhancing the β-galactosidase activity and decreasing the GFP fluorescence.

It turned out that the construction of the bicistronic operon resulted in an unexpected pattern concerning the synthesis of both BgaB and GFP, therefore being not suitable to investigate the role of transcriptional terminators in *B. subtilis*. The plasmid pKL03 seems to show an unexpected structural instability regarding the expression of both *bgaB* and *gfp* in *E. coli*.

Moreover, the construction of the bicistronic operon resulted in a considerable lower BgaB activity and an unpredicted lack of *gfp* expression in *B. subtilis*.

Based on the results obtained on the restriction enzyme analyses, it seems that the plasmid pKL03 does not present a visible structural instability due to rearrangements within the plasmid. Despite this result, it is important to mention that some small deletions or insertions of sequences could have occurred within the plasmid, although at a small frequency that was not able to be detected however it was enough to cause the unexpected pattern of gene expression.

In order to improve a screen for 3'-stabilizing elements, I suggest for further studies integration of the reporter genes into the chromosome to eliminate plasmid copy number effects, which are likely and might cause a gene dosis effect. Furthermore, I recommend using a RNA that has no good 3' end protection. Knowing that in bacteria, mRNA degradation appears to be initiated by one or more endonucleases followed by digestion by 3' to 5' exonucleases (Steege, 2000), the contribution of a 3'-end with a certain level of protection would be sufficient to protect against rapid 3' exonucleolytic decay. A better level of protection could also either increase promoter activity or change the coding sequence that results in a higher enzymatic activity. It is also important to mention that a low level of 3'-end protection also implies a low level of transcription termination, since both are dependent on the 3'-stem-loop structure (Bechhofer *et al.*, 2008). Therefore, a careful analysis of the terminator stability is primary essential as well as the performance of the reporter gene.

4.2 Secretion stress and the "quality control" in *B. subtilis*

Although *B. subtilis* is well-known for its high secretion capacity resulting in high extracellular levels of proteins, not every protein secreted accumulates to high levels in the extracellular medium (Bolhuis *et al*., 1999a; Westers *et al*., 2005; Nijland and Kuipers, 2008; Westers et al., 2008). The major obstacle for secretory proteins is that this organism naturally produces high levels of extracellular proteases that cause extensive protein degradation due to either incorrect

folding or to the presence of exposed protease recognition sequences in the folded protein (Simonen and Palva, 1003; Westers *et al*, 2004).

These proteases belong to the system called ‘quality control’ in *B. subtilis*. The quality control is composed by three key enzymes: two membrane-bound serine proteases, named HtrA and HtrB, and the wall-associated protein A, WprA. These proteases are necessary to ensure that secretory proteins do not block the Sec translocase or interact with the cell wall growth sites (Harwood and Cranenburgh, 2008; Pohl and Harwood, 2010).

The genes encoding these proteins are induced in response to secretion stress and heat shock that might negatively influence the structure of secretory proteins. The induction starts upon detection of misfolded protein by the CssR–CssS two-component signal transduction pathway localized at the interface between the membrane and the cell wall. The resulting induction of HtrA and HtrB reduces the potential for misfolded proteins to block the translocase and/or cell wall growth sites. The signal responsible for the induction of WprA is still unknown (Pohl and Harwood, 2010).

However, not only the quality control proteases are a barrier to the production of secreted heterologous proteins. *B. subtilis* encodes genes for seven so-called ‘feeding proteases’, namely *nprB, aprE, epr, bpr, nprE, mpr, vpr*. These proteases provide sources of nutrients in the environment. Different from quality control proteases, which degrade mainly misfolded proteins, the feeding proteases are adapted folded proteins in the culture supernatant (Pohl and Harwood, 2010).

4.2.1 **The high expression level of α-amylase in *B. subtilis* by pKL01**

The *amyQ* gene coding for an α-amylase from *B. amyloliquefaciens* was used here as a reporter gene in the construction of new strains to improve expression and secretion of heterologous proteins in *B. subtilis*. This gene has been broadly used in *B. subtilis* since the late 70’s (Yoneda *et al.*, 1979; Palva, 1982) and over the last four decades *amyQ* and its signal sequence have been

involved in several studies of overproduction and secretion of heterologous proteins in this organism. Moreover, known as a well-secreted protein, α-amylase has also been a model to investigate the role of overproduction of proteins in different processes like translocation and sporulation in *B. subtilis* (van Wely *et al.*, 1998; Lulko *et al.*, 2007; Chen *et al.*, 2009).

Here, I constructed a plasmid with the *amyQ* gene under control of an IPTG-inducible promoter (pKL01) and analyzed it in different strains to check for an improvement of secretion of an active α-amylase into the supernatant of *B. subtilis*. An experiment was performed to evaluate the detailed migration and localization of this protein analyzing different compartments of the cell. Even though this protein is used as model for oversecretion of proteins in *B. subtilis*, it was shown that this protein still accumulates in a high amount in the cytoplasm and at the cytoplasmatic membrane.

Two important scenarios are possible to explain this pattern. Under conditions of high protein concentration some heterologous proteins occur in soluble (cytoplasm) and insoluble (membrane) cell fractions and many of these proteins accumulate as insoluble aggregates known as inclusion bodies (Ventura and Villaverde, 2006). These aggregates occur due to an irregular or incomplete folding process, which impair the secretion of proteins through the Sec pathway. Almost all parameters that determine protein aggregation have been well known and different factors including co-expression of chaperones have been shown to manipulate the solubility of protein aggregates (Wu *et al.*, 1998; Fahnert *et al.*, 2004; Villaverde and Carrio, 2003).

Another explanation to an unsuccessful translocation of α-amylase into the supernatant is that an overproduction of a heterologous protein can jam the translocation machinery, composed of SecA, SecYEG and other proteins indirectly involved with the translocation process (Breukink, 2009). Regarding this step of the secretion process in bacteria, studies have shown that an overproduction of different components of the translocation machinery and even different subunits of the SecYEG translocon can interfere during secretion of proteins. For instance, the increase of SecA has been reported to enhance the yield of secreted levansucrase in *B. subtilis* cells (Leloup *et al.*, 1999), the expression of *B. subtilis secG* has been shown to be essential and

for the translocation of the precursor of the *B. subtilis* alkaline phosphatase in *E. coli* cells (Swaving *et al.*, 1999). Furthermore, knowing that FtsH, a membrane embedded protease that functions in the quality control of membrane proteins, degrades SecY when the translocon gets jammed, van Stelten has shown that the overexpression of YccA, a substrate for FtsH, led to relieved jamming of the Sec translocon in *E. coli* (van Stelten *et al.*, 2009).

4.3 The co-expression of SecA and α-amylase

From both scenarios discussed above, I decided to work with proteins involved in the translocation machinery in two independent ways: (i) by overexpressing SecA, and (ii) by expressing an artificial *secYEG* operon.

In prokaryotic cells, SecA functions as a motor protein involved in both processes of preprotein translocation and insertion of membrane protein domains utilizing the energy of ATP-hydrolysis to guide these proteins to the translocon and drive the translocation across the cytoplasmatic membrane (Hartl *et al.*, 1990; Randall and Henzl, 2010). Expression of *secA* has been previously addressed to various proteins showing that there are major differences in their dependency on the amount of SecA in the cell (Akita *et al.*, 1990; Lill *et al.*, 1990; Qi and Bernstein, 1999; Duong, 2003; Karamyshev and Johnson, 2005; Papanikou *et al.*, 2005; Scheuring *et al.*, 2005; Zimmer and Rapoport, 2009).

It was already shown that *amyQ* secretion is relatively unresponsive to a decrease in the SecA level in *B. subtilis* cells (Leloup *et al.*, 1999) and our data indicate that the full production of α-amylase is reduced by the co-production and overexpression of SecA. Since SecA is involved in the initial steps of the protein translocation process directly recognizing the signal peptide of the preprotein (Kumamoto and Beckwith, 1985; Tjalsma *et al.*, 1998; Fekkes and Driessen, 1999; Rapoport, 2007; Zimmer and Rapoport, 2009; Yuan *et al.*, 2010) this response of α-amylase to the increase of SecA may be due to the lack of affinity of its signal sequence for SecA (Leloup *et al.*, 1999).

This result also suggests that the translocation of AmyQ may not be Sec-dependent. In *E coli* cells, the preproteins translocated by the post-translational translocation Sec-pathway (Yamane *et al.*, 2004; Harwood and Cranenburgh, 2008; Driessen and Nouwen, 2008), require the recognition and binding of the molecular chaperone SecB to ensure the translocation-competent state of the protein, and this complex is then targeted to SecA to initiate the translocation process (Kumamoto and Beckwith, 1983; Kumamoto and Beckwith, 1985; Hartl *et al.*, 1990; Fekkes *et al.*, 1997; Fekkes and Driessen, 1999; Miller *et al.*, 2002; Zhou and Xu, 2005; Randall and Henzl, 2010).

So far, no SecB homologue has been identified in *B. subtilis*, a finding that raises the question whether translocation of preproteins in this organism is rather co-translational. Even though a gene designated *csaA* has been described to possibly act as a chaperone to prevent folding of polypeptide chains to be translocated (Müller *et al.*, 2000; Shapova and Paetzel, 2001), the evidence for its role in secretion needs to be confirmed experimentally. Although, it is important to mention that CsaA has been shown to interact with SecA, to bind peptides and it is upregulated under secretion stress (Linde *et al.*, 2003; Müller *et al*. 1992; Vitikainen et al, 2005).

To date, the best candidate to be the functional counterpart of SecB in *B. subtilis* is the SRP. It has been shown that the SecA-SRP interaction has only been required to insert membrane proteins into the cytoplasmatic membrane and few works has been performed on the interaction of SRP and SecA for secretory proteins (Bunai *et al.*, 1999; Hirose *et al.*, 2000; Yamane *et al.*, 2004). Recently, Kuhn and colleagues controversially showed that the preproteins target to SRP- and Sec pathways compete for access to the same binding site of the SecYEG translocon (Kuhn *et al.*, 2011). Therefore, one could argue that there are proteins that are either Sec- or SRP-dependent (Fig. 28 – Pohl and Harwood, 2010).

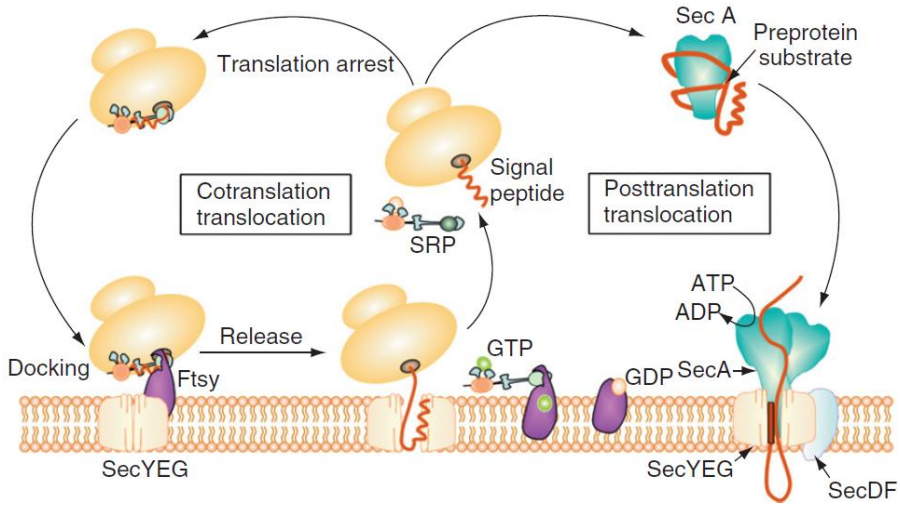

Figure 28: Diagrammatic representation of the targeting pathways of *B. subtilis*. SRP pathway (left side): cotranslational translocation. Sec pathway (right side): posttranslational translocation (Pohl and Harwood, 2010).

Given the significant decrease of α-amylase when SecA is overexpressed, the results shown here may suggest that there is a competition between both overexpressed proteins, the cellular proteins on one hand and SecA on the other hand. Here, one could argue that some of the cellular proteins to be translocated are essential. Furthermore, the interaction of α-amylase with SecA may be decreased because secretion of AmyQ may not only depend on the affinity of its signal sequence for SecA, but may also indicate that translocation of this protein is rather independent of SecA, therefore is more likely to use a co-translational translocation pathway. In order to emphasize this suggestion, further experiments on the analysis of the modulation of SRP and its receptor in *B. subtilis* strains that overexpress α-amylase, such as KL01 have to be carried out.

4.4 Overexpression of *secY*, *secE* and *secG* and their effect on α-amylase secretion in *B. subtilis*

In bacteria, the preproteins are translocated across the cytoplasmic membrane by a protein-conducting channel named as translocon, a heterotrimeric integral membrane protein complex composed of the three subunits SecY, SecE and SecG (Breyton *et al.*, 2002; Boy and Koch, 2009; Mandon *et al.*, 2009; du Plessis *et al.*, 2010). Detailed structure of each component (Murphy and Beckwith, 1994; Homma *et al.*, 1997), their interaction and dependency among the three subunits and with other components of the translocation machinery as SecA and SRP

(Homma *et al.*, 1997; Valent *et al.*, 1998; Zimmer *et al.*, 2008; Boy and Koch, 2009), and also their localization in the cytoplasmatic membrane of bacterial cells as well as their functional mechanism of translocation (Shiomi *et al.*, 2006; Driessen and Nouwen, 2008) have been well described in the literature. In addition, data concerning the interaction between different components of the machinery in heterologous complexes are available. For example, the *B. subtilis* alkaline phosphatase is only translocated in *E. coli* in the presence of the *B. subtilis* SecYEG components (Swaving *et al.*, 1999), the *E. coli* SecA does not complement the *B. subtilis secA* mutant (Takamatsu *et al.*, 1992) as well as the *B. subtilis* SecG and SecDF are unable to replace function of the correspondent mutant proteins in *E. coli* cells (Bolhuis *et al.*, 1998).

Although plenty of information regarding the translocon SecYEG in bacteria has been available, for the first time, I report here that an increase of these translocon components interferes with secretion of proteins in *B. subtilis*. An artificial *secYEG* operon fused to a xylose-inducible operon composed of the *B. subtilis secY, secE* and *secG* genes translated from their own Shine-Dalgarno sequence was integrated into the chromosomal DNA and it was verified that each independent gene was overproduced upon induction. The analysis of the expression and secretion of α-amylase by KL03/pKL01 clearly showed that the newly constructed strain reduces the amount of protein remaining inside of the cytoplasm and attached to the membrane, and, consequently, enhances the quantity of active protein secreted into the culture supernatant.

I propose that the increased expression of the *secYEG* operon coding for the SecYEG translocon enhances the secretion of AmyQ by increasing the amount of translocons in the cytoplasmic membrane of the *B. subtilis* strain KL03. Since the analysis of the supernatant fraction did not indicate cell disruption (there were no changes in both the cytoplasmic and the membrane loading controls), it can be concluded that the cells were not damaged by overexpression of α-amylase after a long period of incubation (12 h). This cell stability is required for the production of the SecYEG components present in the artificial operon in view of the fact that the presence of functional SecYEG is requested for the newly synthesized components (Breukink, 2009).

One could ask the question "How does this construction not allow also the release of more proteases into the supernatant that could rapidly degrade the reporter protein?" In fact, this degradation does not occur because the α-amylase shows a high stability level. This hypothesis is supported by the evidence that *amyE* from *B. subtilis* is spontaneously stabilized in an intermediate folding state since its processing in the cytoplasm (Leloup *et al.*, 1999; Liu *et al.*, 2010). Furthermore, the enhancement of transcription of the genes coding for the translocon can also improve secretion of proteins involved in the folding process such as the chaperone PrsA, that may increase the stability of the α-amylase in the supernatant by aiding its folding (Vitikainen *et al.*, 2001).

Hence, future studies should reveal whether the strain KL03 presents a higher amount of proteases and foldases into the supernatant and how secretion of such proteins is related to the increase of *secYEG* genes, and moreover, how this can interfere with secretion of homologous and heterologous proteins in *B. subtilis*.

4.5 The transposon containing a xylose expression cassette can allow activation of genes in *B. subtilis*

Transposons, also named mobile genetic segments, can produce mutations in the chromosomal genome of an organism by moving from one position to another, a process referred to as transposition. Transposon mutagenesis has been a powerful and broadly used tool to establish whether certain genes are essential in different environments as well as to define their function on the genome of a determined organism. Transposition events result in the creation of knockout strains by generation of insertion mutations that disrupts chromosomal genes (Fig. 29) (Le Breton *et al.*, 2006; Reznikoff and Winterberg, 2008; Choi and Kim, 2009).

The structure of a transposon is defined by transposon-specific terminal DNA sequences that are inverted versions of the same sequences, and only within natural transposons, there is also a gene encoding the transposon-specific transposase. For research purposes, the use of transposition as

genetic tool to introduce insertion mutations into a target organism makes use of plasmid transformation of vectors that contain the transposase encoded by a gene located outside of the transposon structure. This type of construction characterizes a transposition strategy known as "suicide vectors", the same used in this work. Suicide vectors are not able to replicate stably in the receptor organism after having carried out a transposition event under certain experimental conditions. This property guarantees the loss of the suicide vector, therefore also the loss of the transposase gene that turns out to ensure the genetically stability of the transposon. Another characteristic of this type of transposon is the presence of a gene that codes for a certain antibiotic resistance to allow for selection of the transposition events (Reznikoff and Winterberg, 2008).

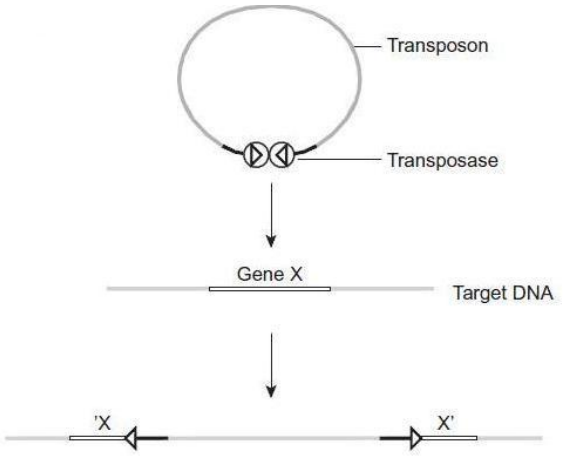

Figure 29: Transposon-mediated gene knockout
The DNA transposon is excised by a transposase and bound to target DNA (gene X). The transposase catalyzes integration of the transposon into gene X thus generating 'X and X' sequences. The transposase is presented as a bold circle. The specific end DNA sequences of the transposon are presented as open triangles (Reznikoff and Winterberg, 2008).

The transposon Tn*10*-based knockout follows the mechanism described above and has been modified for use in *B. subtilis* (Petit *et al.*, 1990). A *mariner*-based knockout performing the same mechanism was also applied successfully and even more productive than the Tn*10* transposon in this organism (Le Breton *et al.*, 2006). While the mariner-*based* transposon is considered available for random mutagenesis, the Tn*10* has hot-spots of insertion (Halling and Kleckner, 1982).

In this work, a transposon-delivery plasmid consisting of the *mariner*-based transposon, TnYLB-1, containing a xylose-inducible promoter fused to it (pKL13), was used in order to activate the expression of the promoter-less *cat* gene.

From the results presented here, one explanation for the expression of the *cat* gene in strain KL07 before transformation of the plasmid pKL13 may be that this gene is expressed under the control of the promoter of the *thrC* locus where it was inserted. Thus, this strain was excluded for further analysis. The strains KL04, KL05 and KL06 developed Cm^r colonies only after transformation of pKL13 and subsequent transposon mutagenesis and less than 10 % of the Cm^r colonies could grow in the absence of xylose. On the other hand, during analysis of the transposition insertion sites, only colonies selected from the strain KL04/pKL13 showed amplification products.

This result indicates that Cm^r clones that grew in the other constructions may reflect subpopulations of cells that had become chloramphenicol-resistant after transposition events. One justification is that the transposon had inserted at a portion of the chromosomal DNA causing a mutation that allowed a certain promoter, inactivated before the transposon mutagenesis, to express this gene. Or, more likely, the only chromosomal structure that allowed insertion of transposon near the Shine-Dalgarno sequence of *cat* was when this gene was inserted in the one orientation within the *amyE* locus. Therefore, a screen of different orientations of the reporter gene as well as diverse integration loci have to be considered in order to identify transposon candidates.

Different from what has been observed by Le Breton and colleagues (Le Breton *et al.*, 2006), the sequence data showed that the transposon had inserted at "GT" dinucleotides, instead of "TA" dinucleotide sites. Moreover, the alignment of the sequences highlights a preference for "A" nucleotide seven bases downstream of the transposon insertion sites, while in the previous study, the same transposon suggested preference for the dinucleotide "TA" five bases downstream and for "T" or "A" five bases upstream the insertion site.

To finalize, I would like to emphasize that the use of *mariner*-based transposon is an efficient approach for identification of random transposon mutants in *B. subtilis*. I also call attention to the fact that it is shown here for the first time that the transposon mutagenesis strategy is not only able to knockout genes, but also to allow expression of genes in *B. subtilis*. The construction and function of modified transposons containing a promoter has only been twice described in the literature in the late ‘80s and for *E. coli*, reported as Tn*5*tac1 composed by a IPTG-inducible promoter (Chow and Berg, 1988) and Tn*5*seq1 composed by both T7 and SP6 (Nag *et al*. 1988).

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List of abbreviations

Abbreviation	Denotation
A_{420}	absorption at a wavelength of 2420 nm
Amp	Ampicillin
amyQ	Gene coding for protein α-amylase (AmyQ)
ATP	Adenosine 5`-triphosphate
α	Alpha, indicating antibodies against; and is the symbol for alpha-amylase
B.	*Bacillus*
bgaB	β-gaclactosidase (BgaB) in *G. stearothermophilus*
bp	Base pair
BSA	Bovine serum albumin
cat	Gene coding for chloraphenicol-acetytransferase
celA	Gene coding for cellulase A (CelA) from *C. thermocellum*
Cm	Chloramphenicol
C. thermocellum	*Clostridium thermocellum*
° C	degrees centigrade
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DnaK	Molecular chaperone DnaK from B. subtilis
dNTP	Desoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylendiamine tetra acetic acid
Erm	Erythromycin
^{R}	Resistantance
et al.	*et alteri*
Ffh	Fifty four homologue
Fig	Figure
g	Gram
GRAS	Generally recognized as safe
G. stearothermophilus	*Geobacillus stearothermophilus*

h	hour(s)
HCl	Hydrocloride acid
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilobase
Km	Kanamycin
LB	Luria-Bertani
lacA	Gene coding for β-galactosidase in *B. subtilis*
l	Litre
M	Molarity (mol/L)
m	Mili
µ	Micro
min	Minute(s)
MOPS	Morpholiopropanesulfonic acid
Neo	Neomycin
nm	Nanometer
OD_{578}	Optical Density at a wavelength of 578 nm
PAGE	Polyacrylamid gel electrophoresis
PCR	Polymerase chain reaction
P*grac*	IPTG inducble promoter, a hybrid promoter of P*groES* and *lac* operator
pmol	picomole
P*spac*	IPTG-inducible promoter, a hybrid promoter of the phage SPO-1 and the *lacO*
P*xylA*	Promoter of *xylA* gene, an xylose-inducible promoter
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Rounds per minute
s	Sensitivity
Sec	Secretion pathway
sec	Seconds
SDS	Sodium dodecyl sulphate
SP	Signal peptide
SRP	Signal recognition particle

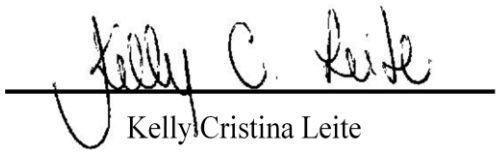
t	Time
Tab	Table
TCA	Trichlor acetic acid
Tet	Tetracycline
TM(S)	Transmembrane (segment)
Tris	Tri-(hydroxymethyl)-aminomethane
Tween-20	Polyoxyethylensorbitane monlaurate
u	Units
V	Volt
v/v	Volume per volume
w/v	Weight per volume

Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, März 2011



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