Studies on the role of SecA, FtsY and Ffh in the insertion of membrane proteins in *Escherichia coli*

A thesis submitted to the University of Wales for the degree of a Doctor of Philosophy

by

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For Wally, Leida, Wendy, Alois and Stephan

L' éléphant et l'Escherichia coli, décembre 1972

„Tout ce qui est vrai pour le Colibacille est vrai pour l'éléphant"

(Jacques Monod)

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1 Abstract

The biogenesis of inner membrane proteins in *Escherichia coli* is a topic of intensive research and evidence is accumulating that these proteins can be targeted and inserted into the membrane via different pathways. However, details of membrane protein biogenesis are still far from understood. The bacterial signal recognition particle (SRP), consisting of the Ffh protein and the 4.5S RNA, and its membrane-associated receptor FtsY are supposed to play an important role in the assembly of numerous inner membrane proteins. A co-operation between the co-translational SRP pathway and SecA, the central component of the post-translational secretion pathway used by periplasmic and outer membrane proteins is less defined.

In order to investigate SecA requirement for inner membrane protein insertion, a seven amino acid cleavage sequence (PCS) that is specifically recognised by tobacco etch virus (TEV) protease was introduced into the *secA* sequence. It was shown in our laboratory that two SecA derivatives could be proteolysed by TEV protease (Mondigler and Ehrmann, 1996). Here, the method of site-specific proteolysis was further improved by preventing autoregulation of SecA and increasing protease accessibility via cleavage of nascent SecA-chains. *lac* promoter controlled *secA*-constructs with the PCS inserted behind amino acids 195, 252 and 830 were integrated into the chromosome at the λ attachment site via phage λ InCh (Boyd *et al.*, 2000). Chromosomal wild-type *secA* was then eliminated by using the λ Red recombinase system (Datsenko and Wanner, 2000). Cleavage of SecA195 by a trigger factor-TEV protease hybrid protein (TF144-TEV protease), that facilitated cleavage of nascent SecA195 at the exit site of the ribosome, resulted in effective inhibition of SecA activity and in a growth defect of the cells. In addition, SecA830 under the control of the *lac* promoter could be depleted in cells not supplemented with the inducer IPTG.

Biotinylation assays showed that inactivation of SecA by site-specific proteolysis or depletion of SecA not only affected the proper secretion of the periplasmic protein alkaline phosphatase, but also the proper biogenesis of the inner membrane proteins FtsQ and MalF274 (consisting of the first three transmembrane domains and the preceding

second periplasmic loop). Similar experiments with Ffh- and FtsY-depletion strains confirmed the involvement of these proteins in inner membrane protein assembly.
DNA macroarray technique was used to compare gene expression profiles of cells depleted either of Ffh, FtsY or SecA with the profiles obtained from un-depleted cells. Upon Ffh-depletion 17 genes were significantly modulated with a false discovery rate (FDR) of 5.9%. FstY-depletion generated gene expression changes of 38 genes with a FDR of 6.5%, whereas when SecA was depleted gene expression of 161 genes changed significantly with a FDR of 6%. Interestingly, while depletion of Ffh and FtsY resulted in the induction several heat shock genes, depletion of SecA induced the expression of another group of stress-response genes, the *psp* operon. The gene products of this operon, the phage shock proteins, have been linked to a protective role in various stressful conditions, including the infection of filamentous phages, heat, ethanol treatment and blockage of protein export (Model *et al.*, 1997; Kleerebezem and Tommassen, 1993). In addition, among the up-regulated genes in SecA-depleted cells was *msyB*, a gene previously identified as a suppressor of SecY24 mutants (Ueguchi and Ito, 1992).

Taken together, it was shown here that site-specific proteolysis could be used to inhibit the activity of an essential protein *in vivo*. Our results obtained by inhibition of SecA activity strengthen the notion that SecA not only plays the central role in post-translational secretion of preproteins, but is also extensively involved in co-translational protein translocation. In addition, DNA array analysis indicated a possible involvement of cellular chaperones in targeting of inner membrane proteins.

2 Introduction

2.1 The cell envelope of *Escherichia coli*

In 1886 Theodor Escherich isolated for the first time a bacterium which turned out to be part of the normal flora of vertebrates intestinal tract: *Escherichia coli*, a member of the family of Enterobacteriaceae (Fig. 2.1).

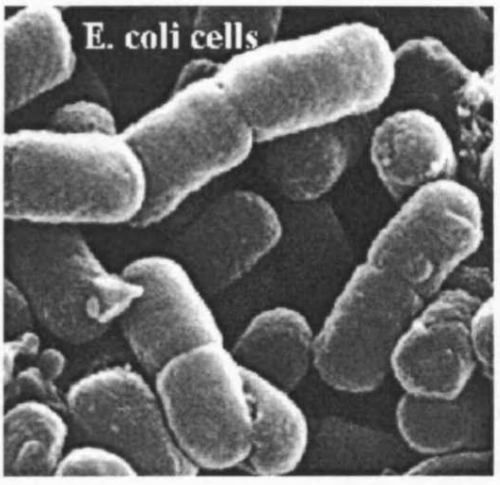


Fig. 2.1 Electron micrograph of growing *E. coli* cells. The cells are approx. 2 µm long and 0.5-1.0 µm wide. Image from http://www.slic2.wsu.edu:82/hurlbert/micro101/pages/Chap.2html#two bact groups. © Dr. R. E. Hurlbert, 1999.

On the basis of its response to Gram stain *E. coli* belongs to the group of gram-negative bacteria. This stain was developed by Christian Gram in 1884 and differentiates between two types of bacteria because of differences in composition and structure of the bacterial cell wall. The gram-negative cell wall consists of the outer membrane, the periplasm and a thin peptidoglycan layer. Together with the plasma membrane the cell wall forms the bacterial cell envelope (Fig. 2.2), which not only provides mechanical protection of the cytoplasm but also has essential physiological functions (Beveridge, 1999).

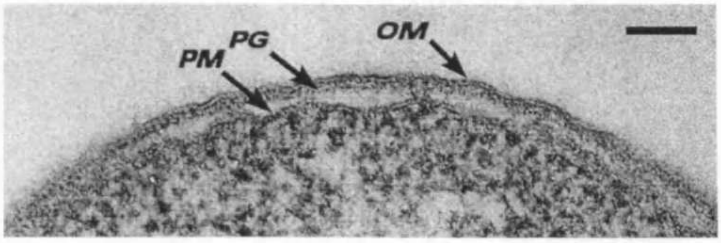


Fig. 2.2 Cell envelope of *E. coli* K-12 (Beveridge, 1999). Abbreviations: OM = outer membrane, PG = peptidoglycan layer, PM = plasma membrane. Bar =100nm

2.1.1 The outer membrane

In contrast to most biological membranes the outer membrane forms a rather atypical bilayer. It consists of lipopolysaccharides (LPSs), phospholipids and proteins such as hydrophilic channel proteins (porins) and lipoproteins (Fig. 2.3). One of its unusual characteristics is the asymmetric distribution of lipids over its inner and outer leaflet. The outer leaflet of the bilayer contains all LPS molecules whereas phospholipids are found predominantly in the inner leaflet (Muehlradt and Golecki, 1975).

The O-antigen polysaccharide region of the LPS protrudes into the medium and is highly variable. It plays an important role in virulence because the O-polysaccharide is responsible for resistance against phagocytosis (Nikaido, 1996).

The outer membrane retains periplasmic compounds that are important for cell viability. However, the cell also relies on the permeability of the outer membrane for the uptake of nutrition and the export of wastes. These tasks are performed by only a few different species of proteins. The most abundant proteins in the outer membrane are a number of trimeric porins. They can represent up to 2% of the total cell-protein (Nikaido, 1996). Most porins form relatively unspecific pores (Benz, 1988) and allow the diffusion of small hydrophilic molecules of up to about 700 Daltons. Bulky nutrients like vitamin B12 are imported via energy-dependent receptors. A number of channels are specific for a certain substrate, for example the specific porin LamB that imports maltodextrins (Lazdunski *et al.*, 1998).

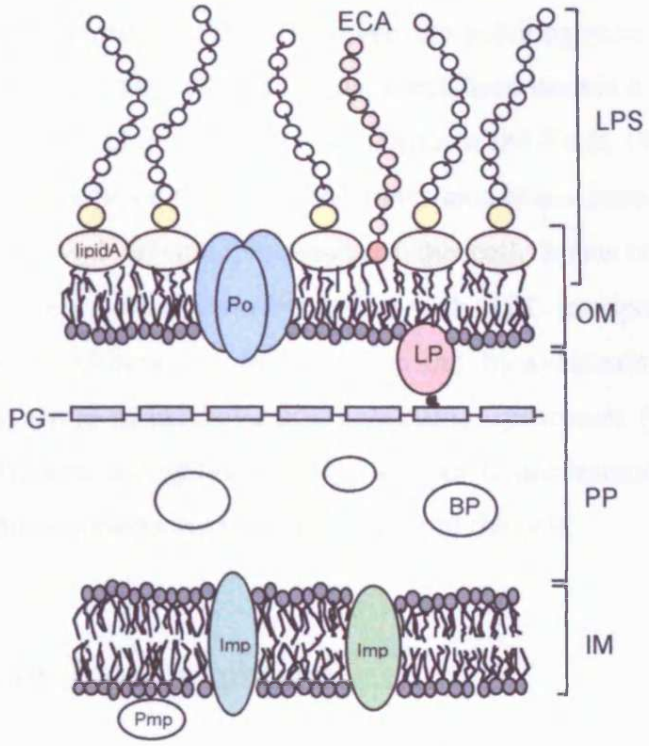


Fig. 2.3 Structure of the gram-negative cell envelope. Abbreviations: BP = binding protein; ECA = enterobacterial common antigen; IM = inner membrane; Imp = integral membrane protein; LP = lipoprotein; LPS = lipopolysaccharide; OM = outer membrane; PG = peptidoglycan; Pmp = peripheral membrane protein; Po = porin; PP = periplasm (Schlegel, 1992; Rick and Silver, 1996).

Although the outer membrane is associated with the underlying peptidoglycan-layer via the lipoproteins many of the bilayer constituents are fluid and in continual rapid motion (Beveridge, 1999).

2.1.2 The murein sacculus and the periplasm

The murein sacculus of *E. coli* is located in the periplasmic space underneath the outer membrane. It maintains the shape of the cell and is probably made of only one layer of peptidoglycan (also called murein) with a thickness of about 7 nm (Park, 1996). The peptidoglycan forms a characteristic meshwork of glycan strands which are cross-linked via peptide bridges (Hoeltje, 1998). The sacculus is rather flexible due to the peptide

bridges which can be stretched to fourfold the length in their most compact conformation (Isaac and Ware, 1974; Hoeltje, 1998). However, the peptidoglycan meshwork forms a barrier for proteins of more than 50 kDa. Thus, specialised murein hydrolases help with the transenvelope transport of globular proteins (Dijkstra and Keck, 1996).

The periplasmic space between the outer and inner membrane contains many different proteins that are involved in vital processes of the cell. Some of them function as substrate binding proteins, which work together with ABC transporters and signal or chemotaxis receptors. Others are involved in the biosynthesis of cell envelope compounds or play a role in catabolic and detoxifying processes (Oliver, 1996). The contents of the periplasm is highly dynamic in order to accommodate changes in the environment, and thus it reflects the metabolic status of the cell.

2.1.3 The cytoplasmic membrane

The cytoplasmic membrane (also called plasma membrane or inner membrane) forms the main osmotic barrier of the cell by controlling influx and efflux of substances. Like most biological membranes the inner membrane consists of a phospholipid bilayer with proteins embedded within it. The *E. coli* cytoplasmic membrane contains a wide range of proteins. At least 100 species were identified by two-dimensional polyacrylamide gel-electrophoresis (Sato *et al.*, 1977). Integral membrane proteins are anchored in the membrane by one or several hydrophobic membrane-spanning domains, which are connected by hydrophilic cytoplasmic and periplasmic segments. Other membrane proteins are peripherally bound or only temporarily associated with the membrane. The membrane proteins flow freely in the lipid bilayer, but their distribution is variable in response to different growth conditions. The protein content is so high that lipids and proteins cover almost the same amount of space over the membrane. Membrane proteins are involved in nearly all essential processes like energy generation, transport of ions and nutrients, translocation of proteins and carbohydrates, transmembrane signaling and chemotaxis as well as cell wall assembly (Kadner, 1996).

2.2 Protein Translocation in *E. coli*

The synthesis of all proteins occurs in the cytoplasm. Thus, the cell faces the challenge of translocating proteins that are not active in the cytoplasm to their proper compartment anywhere in the cell envelope or to the extracellular medium. This process involves targeting to the cytoplasmic membrane and secretion across or insertion into the inner or outer membrane.

The mechanism of transferring proteins to their final locations is a highly conserved process among bacteria, archaea and eukaryotes (Schatz and Dobberstein, 1996). Common features are the recognition of targeting signals of the transported protein by targeting factors on the *cis* side of a membrane (i.e. the cytoplasmic side in bacteria), a hetero-oligomeric transmembrane channel which gates across and into the membrane, a peripherally associated energy generating component and a folding system on the *trans* side of the membrane (i.e. the periplasmic side in bacteria).

2.2.1 Translocation systems

Gram-negative bacteria possess at least two major post-translational export systems that translocate proteins across the cytoplasmic membrane. The general export pathway (GEP), also called Sec-dependent pathway (Manting and Driessen, 2000) translocates unstructured proteins, whereas the Tat system (Palmer and Berks, 2003) exports folded proteins into the periplasm. In contrast, the mechanisms by which proteins are secreted into the extracellular environment are rather species- and substrate-specific. At least five different mechanisms are known in gram-negative bacteria (Wandersman, 1996). Some of these mechanisms are dependent on the Sec-machinery, like the most widespread secretion pathway, the general secretion pathway (GSP)(Pugsley, 1993).

2.2.1.1 Export across the cytoplasmic membrane

2.2.1.1.1 The Sec-translocase

The majority of proteins cross the cytoplasmic membrane via the general export pathway to reach their final destination in the periplasm or the outer membrane (Pugsley, 1993). Secretory proteins, also called preproteins, are synthesised with a cleavable N-terminal signal peptide (Fig. 2.4) and are transferred across the inner membrane in an extended conformation by a well-studied protein complex termed translocase (Wickner *et al.*, 1991). The translocase or Sec-machinery consists of at least three integral membrane proteins: SecY, SecE and SecG, and the peripherally bound cytosolic ATPase SecA (Driessen *et al.*, 1998). The cytosolic chaperone SecB keeps preproteins in an export-competent confirmation by preventing the folding of the preprotein prior to its export.

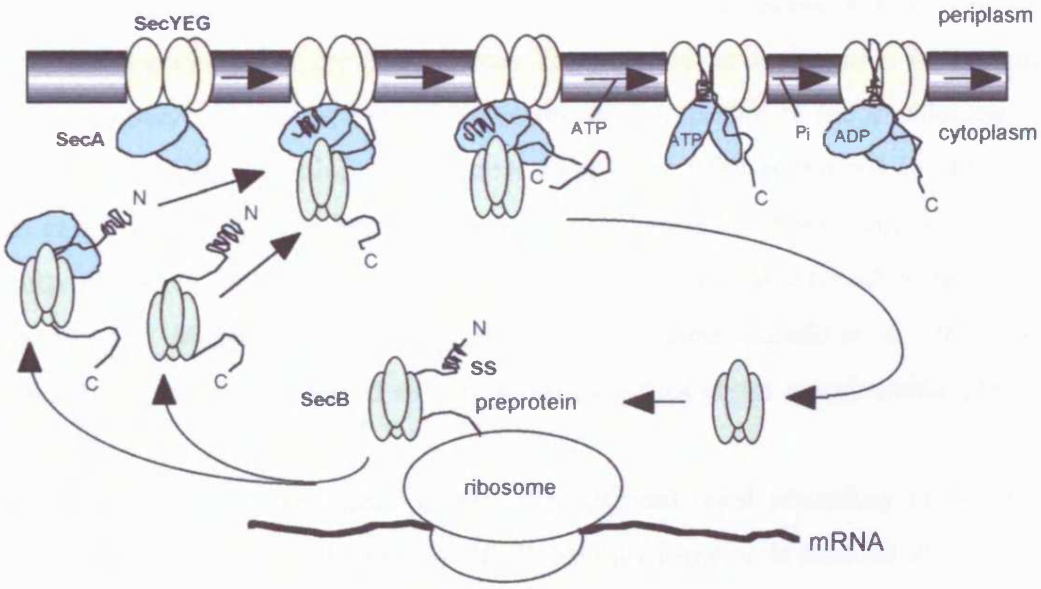


Fig. 2.4 The Sec-dependent translocation pathway. Abbreviations: SS = signal sequence.

The preprotein-SecB-complex is recognised by soluble or membrane associated SecA and thus targeted to the SecYEG translocon. Upon repeated cycles of ATP binding and hydrolysis by SecA the preprotein is pushed stepwise through the channel formed by the SecYEG protein-complex. After crossing the inner membrane the signal peptide is

removed and the secreted protein is folded to either stay in the periplasm or to be targeted to the outer membrane (Danese and Silhavy, 1998).

The SecYEG complex not only exports preproteins into the periplasmic space but also facilitates membrane insertion of proteins that were targeted to the inner membrane by the signal recognition particle and its receptor FtsY (Valent *et al.*, 1998).

The importance of the protein-conducting channel is also evident in the observation that components of the Sec-translocase are highly conserved in evolution and homologues have been found also in archaea, yeast and mammals. The mammalian Sec61p complex forms the translocon of the ER membrane. Its α-and γ-subunits show homology to the *E. coli* SecY and SecE proteins respectively (Hartmann *et al.*, 1994; Rapoport *et al.*, 1996)

2.2.1.1.1.1 The multifunctional ATPase SecA

SecA is a multifunctional and dynamic protein, which plays a key role in the translocation process. All secretory proteins that have to cross the inner membrane are dependent on SecA. However, in contrast to the core structure of the translocase, SecY and SecE, eukaryotic homologues of SecA have only been identified in chloroplasts (Nakai *et al.*, 1994). *E. coli* SecA is a cytoplasmic protein of 901 amino acids and a molecular weight of 102 kDa (Schmidt *et al.*, 1988) that was also found to be associated peripherally and integrally with the cytoplasmic membrane (Cabelli *et al.*, 1991). It was observed that SecA is functional as a homodimer throughout translocation (Driessen, 1993).

The amount of SecA is regulated at the translational level according to the protein secretion status of the cell; when the ability to export proteins is reduced the translation of SecA is increased. Only recently it became evident that the secretion-proficient state of the cell is monitored by the SecM protein (for **sec**retion **m**onitor). *secM* (formerly called gene*X*) is located upstream of *secA* and both genes are co-transcribed (Schmidt *et al.*, 1988). The signal sequence of SecM has an atypical extended N-terminal region containing some aromatic amino acyl residues that were suggested to be critical for proper *secA* regulation (Sarker *et al.*, 2000). This atypical signal peptide might be responsible for co-translational export of SecM into the periplasm, where it has no

obvious function and is rapidly degraded. It was shown that SecM-translocation is dependent on the signal recognition particle, SecA and SecY (Nakatogawa and Ito, 2001). Based on the observation that SecM undergoes a self-translation arrest in the cytoplasm, the authors also suggested a mechanism that couples the secretion status of SecM and SecA translation (Nakatogawa and Ito, 2001). According to this mechanism a pause occurs during SecM translation which is transient in cells with normal translocation activity. Under conditions where translocation is impaired SecM might fold co-translationally into an "arrestase" domain that interacts with the ribosome causing a prolonged translation arrest. The stalled ribosome might then disrupt a secondary structure of the *secM-secA* mRNA. This structure is thought to be a stem-loop termed helix II (McNicholas *et al.*, 1997) that occludes the *secA* Shine-Dalgarno sequence. Upon interaction with the stalled ribosome this sequence might be exposed and *secA* translation is facilitated. In addition to this regulation mechanism soluble cytoplasmic SecA acts as an autorepressor by binding to its translation initiation region. If the translocation process is impaired SecA dissociates from the binding site and translation can occur (Schmidt and Oliver, 1989; Dolan and Oliver, 1991; Salavati and Oliver, 1997).

SecA not only binds to its own mRNA but has a large number of interdependent ligands. Cytoplasmic SecA binds to preproteins either alone or in complex with SecB. SecA recognises the signal peptide as well as the mature part of the protein (Cunningham and Wickner, 1989; Akita *et al.*, 1990). It also interacts directly with SecB. SecA also binds acidic phospholipids, nucleotides and the SecYEG translocon. Recently, the solution of the crystal structure of *Bacillus subtilis* SecA gave some insight into the interdomain packing of soluble SecA and confirmed that SecA undergoes conformational changes upon binding of ligands (Hunt *et al.*, 2002).

Several functional regions of the SecA protein have been proposed (Fig. 2.5.). The carboxy-terminal third is involved in its dimerisation (Hirano *et al.*, 1996) and in SecY interaction (Snyders *et al.*, 1997). However, in another study most SecYE-specific binding activity was found in an N-terminal SecA fragment (SecA-N664) (Dapic and Oliver, 2000). The domain identified for preprotein-interaction is localised between amino acids 267 and 340 with Tyr-326 playing a critical role (Kimura *et al.*, 1991; Kourtz and Oliver, 2000).

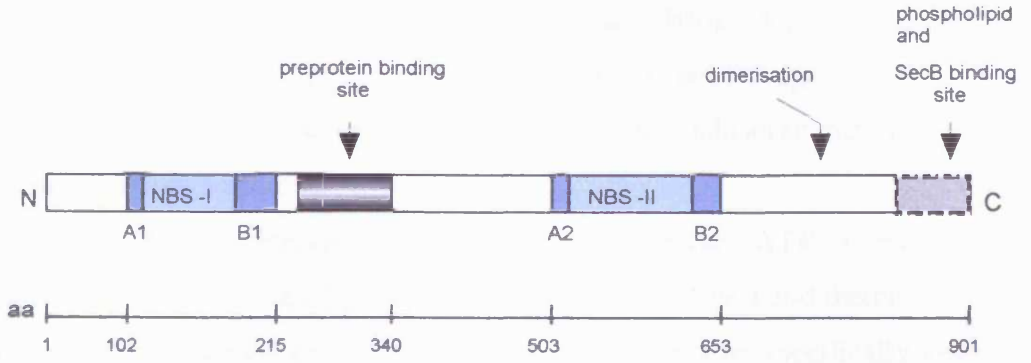


Fig. 2.5 Functional regions on the SecA protein. Abbreviations: aa = amino acid; A = Walker A motif (GXXXXGKT); B = Walker B motif (hXhhD; h = hydrophobic); NBS = nucleotide binding site.

The carboxy-terminal 70 residues form a binding site for phospholipids and SecB (Breukink *et al.*, 1995). The most distal 22 amino acyl residues are sufficient for SecB interaction (Fekkes *et al.*, 1997). This region is rich in arginyl and lysyl residues and therefore positively charged (Fekkes and Driessen, 1999). However, in absence of a preprotein the association between SecA and SecB is of low affinity (Hartl *et al.*, 1990).

Upon binding of ATP SecA is in a conformation which favours its interaction with precursor proteins (Lill *et al.*, 1990). SecA possesses two nucleotide-binding sites (NBSs), which are both essential for translocation. These NBSs show homology to RNA and DNA helicases from superfamilies I and II (Koonin and Gorbalenya, 1992; Caruthers and McKay, 2002). In addition, the seven expected helicase motifs are located in either of the nucleotide binding sites (Mitchell and Oliver, 1993). However, SecA helicase activity is not required for efficient protein translocation and *secA* regulation (Schmidt *et al.*, 2001). The amino-terminal NBS-I has a much higher affinity for ATP ($K_D \approx$ 0.1-0.3 μM) than NBS-II with a K_D of about 300-500 μM (Mitchell and Oliver, 1993; den Blaauwen *et al.*, 1996). These binding sites contain Walker A and B motifs that are characteristic of a major class of nucleotide-binding sites (Walker *et al.*, 1982). NBS-I is important for energising the translocation process and might be conformationally regulated (Schmidt *et*

al., 2000). The function of NBS-II is not clear as *in vitro* translocation can occur with ATP concentrations far below the K_D of NBS-II (Jarosik and Oliver, 1991). However, mutations in NBS-II lower the ATPase activity at NBS-I (Mitchell and Oliver, 1993; Economou *et al.*, 1995). A recent NMR sudy identified mobile regions of SecA located in NBS-II and the C-terminal segment (Chou *et al.*, 2002), indicating that these regions play a role in regulatory functions.

Following ATP- and precursor binding SecA hydrolyses ATP, a reaction which is stimulated by the presence of phospholipids (Lill *et al.*, 1990) and therefore termed lipid ATPase activity (Shinkai *et al.*, 1991). SecA can either non-specifically associate with the phospholipids of the inner leaflet of the membrane or bind to the translocase components SecY and SecE (Hartl *et al.*, 1990). Hydrolysis (Murphy and Beckwith, 1996) or binding of ATP (Economou and Wickner, 1994) may cause a conformational change of SecA (den Blaauwen *et al.*, 1996), which facilitates its association with the membrane and helps the preprotein to insert into the membrane at the translocation site. Upon interaction with the components of the translocon SecA undergoes continuous cycles of ATP binding and hydrolysis. This activity is necessary for preprotein translocation and hence termed translocation ATPase (Cunningham and Wickner, 1989; Lill *et al.*, 1989). It was shown that the preprotein interacts with SecA during various stages of the translocation process (Joly and Wickner, 1993).

The above observations have lead to the following translocation model. According to this model a C-terminal proteolytically stable 30 kDa segment of SecA (Price *et al.*, 1996) would insert into the membrane resulting in the translocation of 20 to 30 amino acids of the preprotein (Economou and Wickner, 1994). Upon ATP hydrolysis SecA would release its substrate and de-insert from the membrane (Economou *et al.*, 1995). The released precursor segment may then be translocated further by the proton motive force (PMF) (Schiebel *et al.*, 1991; Driessen, 1992). Several rounds of ATP-binding and hydrolysis combined with SecA insertion into the membrane and de-insertion would push the precursor through the SecYEG channel in a stepwise manner.

Although the 30 kDa fragment of SecA is protected from proteolysis and shielded probably by the SecYEG channel (Eichler and Wickner, 1997) other findings contradict

the model of SecA membrane insertion (van der Does *et al.*, 1998; Manting and Driessen, 2000). It was also observed that SecY and SecE were not essential for the translocation of all proteins *in vitro* (Yang *et al.*, 1997). A recent electron microscopy study revealed that SecA forms ring like pore structures upon interaction with anionic phospholipids (Wang *et al.*, 2003). The authors suggested that these pores might form the central core of a protein-conducting channel. The size of the pore structures averaged 205 kDa, equivalent to two SecA subunits. Or and coworkers showed that acidic phospholipids caused the dissociation of the SecA dimer (Or *et al.*, 2002). On the basis of their data and of sequence similarities between SecA and certain monomeric helicases they proposed that SecA dissociates into monomers during protein translocation.

These observations provide the scenario for an alternative mechanism of how SecA facilitates preprotein export. It might be possible that two SecA monomers stack on one another forming a pore, probably in conjunction with SecYEG. As monomeric helicases are thought to move along single-stranded nucleic acids by an 'inchworm' mechanism (Soultanas and Wigley, 2000) it may be possible that SecA might translocate a preprotein in a similar manner (Or *et al.*, 2002).

Taken together, the interactions between the subunits of the translocase and especially the mechanism by which SecA allows a precursor protein to cross the inner membrane are still far from understood.

2.2.1.1.1.2 The protein translocation channel

The integral membrane proteins SecY, SecE and SecG provide the structural basis for the channel through which preproteins can cross the inner membrane (Brundage *et al.*, 1992; Simon and Blobel, 1992; Joly *et al.*, 1994). SecY and SecE are essential proteins (Schatz *et al.*, 1989; Nishiyama *et al.*, 1991) (Akimaru *et al.*, 1991) that form a stable complex in the cytoplasmic membrane and upon solubilisation (Brundage *et al.*, 1990). This complex is the minimal constituent of the membrane translocation apparatus (Duong and Wickner, 1997). SecG is co-purified with SecY and SecE (Brundage *et al.*, 1990) but is only essential *in vivo* at low temperatures (Nishiyama *et al.*, 1994). It was shown that SecG is

interchangeable between different SecYE complexes in the cytoplasmic membrane (Joly *et al.*, 1994).

SecY is the largest subunit of the SecYEG heterotrimeric complex with a molecular mass of 48 kDa and 10 membrane spanning domains (Akiyama and Ito, 1987; Ito, 1990).

SecE, a protein of 14 kDa spans the membrane with 3 hydrophobic domains. A conserved minimal form of SecE consisting of the third transmembrane domain, the C-terminal periplasmic- and the second cytoplasmic domain is sufficient for its function (Schatz *et al.*, 1991). Several regions that are involved in interactions between SecY and SecE are identified in both proteins (Baba *et al.*, 1994; Pohlschroder *et al.*, 1996). The ATPase SecA binds to SecY with high affinity. This interaction can be cross-linked *in vivo* (Manting *et al.*, 1997) and multiple sites in SecY may be involved in their association (Matsumoto *et al.*, 1997; Snyders *et al.*, 1997). It was shown that especially the cytoplasmic C-terminal residues of SecY are important for SecA-dependent translocation initiation (Chiba *et al.*, 2002).

SecG (11.5 kDa) traverses the inner membrane twice and possesses a hydrophobic cytoplasmic segment (Nishiyama *et al.*, 1996). Although SecG stimulates translocation *in vitro* and *in vivo* (Nishiyama *et al.*, 1993; Nishiyama *et al.*, 1994) its function remains unclear. Nishiyama and coworkers reported a topology inversion of SecG, which was linked with the membrane insertion and de-insertion cycles of SecA, indicating SecG assistance for SecA function (Nishiyama *et al.*, 1996). Indeed SecG does stimulate SecA insertion *in vitro* (Duong and Wickner, 1997). Another proposed function of SecG is a facilitation of preprotein movement through the membrane either by direct interaction with the preprotein (Bost and Belin, 1997) or in close conjunction with SecA. This cooperation would not only facilitate SecA insertion but also increase the yield of preprotein movement in each cycle (Matsumoto *et al.*, 1998).

Signal sequence suppressor mutations (see also chapter 2.2.2.1) have been identified in all three subunits but direct interactions with a translocating precursor have only been shown for SecY (Joly and Wickner, 1993). The mutant alleles of *secYEG* were termed *prl* genes for ***pr***otein ***l***ocalisation (Stader *et al.*, 1989; Bieker *et al.*, 1990; Schatz and Beckwith, 1990; Osborne and Silhavy, 1993; Bost and Belin, 1997).

The PrlA mutants of SecY allow the passage of preproteins with a defective or even without a signal peptide (Derman *et al.*, 1993; Flower *et al.*, 1994). As a result of these observations a proofreading function for SecY was suggested in a way that SecY would "proof" signal sequences presented by SecA prior to the translocation process (Osborne and Silhavy, 1993). Prl mutants would have lost this function.

Electron microscopic analysis of the purified SecYEG complex revealed a two-channel-like structure, which corresponds to a dimer of SecYEG. An even larger structure composed of four SecYEG complexes was observed upon membrane insertion of SecA or trapping of a precursor in the translocase (Manting *et al.*, 2000). Manting and coworkers suggested that the large SecYEG channel structure dissociates into a dimeric form after the translocation reaction.

The finding that one SecYEG monomer is too small to form a functional channel unless SecA is recruited to form a supercomplex (Collinson *et al.*, 2001) and the observation that SecA forms pore like structures in anionic phospholipid layers (Wang *et al.*, 2003) indicate that SecA might also play an important role in forming the translocation channel.

2.2.1.1.1.3 Accessory translocation components

Several additional membrane proteins are involved in the translocation process. Genetic studies led to the identification of the membrane proteins SecD and SecF that expose large loops into the periplasm (Pogliano and Beckwith, 1994). They are both not essential for translocation under some conditions but null mutants are severely cold sensitive (Pogliano and Beckwith, 1994). SecD and SecF form a complex together with YajC (Duong and Wickner, 1997), and the three corresponding genes are located on the same operon (Pogliano and Beckwith, 1994). Although not clearly defined, their proposed function occurs at a late stage during translocation, as no *prl* mutations have been found in these proteins.

SecD and SecF do not always co-fractionate with the SecYEG complex which indicates that their interaction is either weak or transient.

It has been shown *in vitro* that the SecDFYajC complex stabilises the membrane inserted state of SecA and inhibits further movements in either direction of the precursor protein

(Economou *et al.*, 1995; Duong and Wickner, 1997). Cells depleted of SecD and SecF are not able to maintain a proton motive force (PMF) across the membrane (Arkowitz and Wickner, 1994) and it was suggested that this complex is involved in closing the channel after translocation.

SecD has also been suggested to facilitate folding and/or release of proteins translocated across the membrane from the secretion apparatus (Matsuyama *et al.*, 1993).

A newly identified component of the translocation apparatus is YidC, a 60 kDa protein and a homologue to the mitochondrial Oxa1p protein (Scotti *et al.*, 2000). YidC can be co-purified with the SecYEG complex and cross-linked to transmembrane segments of translocating integral membrane proteins. Therefore, YidC might act as a receptor for signal anchor sequences of integral membrane proteins that have exited the Sec-translocase. Alternatively, YidC may help to open the translocase laterally into the membrane (Scotti *et al.*, 2000).

As soon as the N-terminus of a precursor protein emerges on the periplasmic side the signal peptide is proteolytically removed by leader peptidases (also termed signal peptidases). The processing of most preproteins to their mature forms is mediated by Lep (typeI leader peptidase), whereas prelipoproteins are substrates of LspA (typeII leader peptidase) (Dalbey and von Heijne, 1992; Dalbey *et al.*, 1997). Lep is anchored in the cytoplasmic membrane with two membrane-spanning segments (Wolfe *et al.*, 1983) and has its active site in the periplasm (Bilgin *et al.*, 1990). After cleavage by Lep the signal peptide is degraded further by other proteases.

Taken together, depending on the kind of delivered preprotein, the translocase might recruit different additional components as is the case in yeast (Wilkinson *et al.*, 1997).

2.2.1.1.2 The Tat system

Recently, it was shown that at least a second export pathway across the cytoplasmic membrane exists which operates quite differently from the Sec-machinery. This export pathway was termed Tat system, standing for ***t***win ***a***rginine ***t***ranslocation (Sargent *et al.*, 1998), because its substrates are directed by an N-terminal signal peptide containing a consensus S-R-R-x-F-L-K "twin arginine motif". In contrast to the Sec-machinery the Tat system translocates proteins that are folded in the cytoplasm prior to their export (Berks *et al.*, 2000; Stanley *et al.*, 2000). These are often proteins that bind cofactors in the cytoplasm or require cytoplasmic factors for folding. Even assembled multimeric complexes are translocated by the Tat system with only one signal peptide on one of the subunits (Rodrigue *et al.*, 1999). Four genes encoding components of the Tat pathway have been identified so far (Sargent *et al.*, 1998). All four components are integral inner membrane proteins (Fig. 2.6). TatA, TatB and TatE are sequence-related proteins that consist of a membrane-spanning helix followed by an amphipathic helix in the cytoplasm. TatC is predicted to be a polytopic inner membrane protein with four (Gouffi *et al.*, 2002) or six (Sargent *et al.*, 1998) transmembrane domains. TatB and TatC are essential components of the Tat system (Bogsch *et al.*, 1998) and are thought to form a receptor complex for Tat substrates (Sargent *et al.*, 1999; Bolhuis *et al.*, 2001). As mutations in either *tatA* or *tatE* genes only block the Tat export when combined in a double *tatA tatE* mutant strain it was concluded that the two proteins have overlapping functions (Sargent *et al.*, 1998) and it was suggested that *tatE* is a cryptic gene duplication of *tatA* (Jack *et al.*, 2001). The suggested role for TatA is the formation of the transport channel (Palmer and Berks, 2003). TatA is present in high molar excess over TatB and TatC (Jack *et al.*, 2001) and negative-stain electron microscopy of a TatAB complex containing predominantly TatA revealed an annular pore like structure (Sargent *et al.*, 2001). Therefore, several TatA helices might form a gated aqueous channel across the membrane.

Several observations indicate the presence of substrate-specific "proofreading" chaperones, at least for some Tat substrates (Pommier *et al.*, 1998; Oresnik *et al.*, 2001). These chaperones might serve to shelter the signal peptide against recognition by Tat

components at an early stage and simultaneously keep the mature protein competent for cofactor insertion.

The bacterial Tat system is closely related to the plant thylakoid ΔpH-dependent import system (Settles *et al.*, 1997) and the translocation is exclusively driven by the proton motive force (Alami *et al.*, 2002). Still, not much is known about the precise role of each Tat component and how the cell manages the challenge of exporting folded proteins or even enzyme complexes of variable sizes across the cytoplasmic membrane without making the membrane permeable to ions.

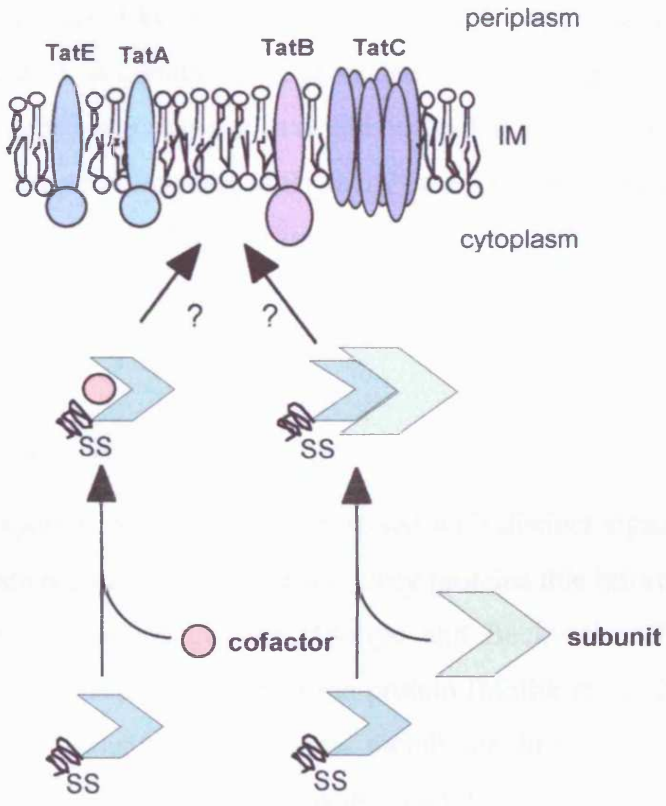


Fig. 2.6 Components of the Tat system in the inner membrane and Tat substrates in the cytoplasm. Abbreviations: SS = signal sequence.

2.2.1.2 Export across the outer membrane

Gram-positive bacteria use the signal peptide-dependent general export pathway to release proteins into the external medium. In gram-negative bacteria the secretion mechanisms are more various. The widely used laboratory strain *E. coli* K-12 does not secrete chromosome-encoded proteins beyond the outer membrane. However, in pathogenic *E. coli* strains the secretion mechanism for extracellular factors such as degradative enzymes or toxins is aside from a few special cases not dependent on the Sec-machinery. The crossing of both membranes without a periplasmic intermediate requires a different set of proteins: ABC proteins, membrane fusion proteins in the inner membrane and a specific outer membrane component, e.g. TolC (Wandersmann, 1996; Binet *et al.*, 1997). Proteins secreted by this so-called type I pathway are usually high-molecular-weight toxins, like the α-hemolysin of *E. coli* (Cavalieri *et al.*, 1984; Gentschev *et al.*, 2001). A common Sec-dependent secretion pathway in Gram-negative bacteria (except *E. coli*) is a two-step mechanism called the general secretory pathway (Pugsley, 1993). Substrates cross the inner membrane via the Sec-translocon or even the Tat system (Voulhoux *et al.*, 2001). Translocation across the outer membrane is then performed by the so-called type II pathway consisting of 12 to 14 specific proteins.

2.2.2 Targeting signals

Precursors of transported proteins are synthesised with distinct signals that direct them along the translocation pathway. In most secretory proteins this information is located in a cleavable N-terminal signal peptide (Inouye and Beckwith, 1977) as well as in unknown sequences in the mature part of the protein (Mallik *et al.*, 2002). Cytoplasmic membrane proteins are anchored in the inner membrane through hydrophobic α-helical stretches that function as internal non-cleavable signal sequences. They are also termed stop-transfer signals. Only the periplasmic loops of such proteins have to cross the inner membrane. The secretion signals for proteins that are released into the external medium via the type I pathway are located in C-terminal non-cleavable sequences (Felmlee *et al.*, 1985; Mackman *et al.*, 1987).

2.2.2.1 *Structure and function of the signal peptides*

The signal peptide (also termed signal sequence) provides efficiency and speed for the translocation process (Derman *et al.*, 1993). It functions as an export signal and plays a role in directing an exported protein into a distinct targeting and translocation pathway. This is evident in the observation that precursor proteins accumulate in the cytoplasm in strains with mutated Sec-pathway signal sequences (Bedouelle *et al.*, 1980; Michaelis *et al.*, 1983). The signal peptide is thought to be recognised by components of the translocase and it was suggested that the presence of the signal sequence alters the folding properties of the mature part of the preprotein, and therefore allows the binding of targeting factors like SecB (Park *et al.*, 1988; Laminet and Pluckthun, 1989).

Not much is known about the nature and chronology of interactions between the signal peptide and the components of the Sec-pathway or the mode of insertion of the signal peptide into the membrane during translocation. Interactions of the signal peptide with the components of the secretion machinery have been concluded from the observation that mutations in *secA*, *secY*, *secE* and *secG* (*prl* alleles) can suppress translocation blockade caused by a defective signal sequence. It is thought that SecY and SecE function in proofreading of signal sequences, not allowing the translocation of defective preproteins. All *prlA/secY* and *prlG/secE* alleles examined suppressed all kinds of signal peptide mutations and even complete deletions of signal peptides (Derman *et al.*, 1993; Flower *et al.*, 1994; Prinz *et al.*, 1996). These observations led to the conclusion that PrlA and PrlG suppressors lost their ability to recognise signal peptides or to interact with them (Flower *et al.*, 1994). In *prlA* and *prlG* suppressor strains the translocation of preproteins with signal sequence mutations is totally dependent on cytoplasmic targeting factors like SecB, even if the protein is normally not dependent on SecB, e.g. alkaline phosphatase (Derman *et al.*, 1993). SecB binds to the mature part of the protein late during or after translation. Therefore, the critical step in translocation of signal peptide defective proteins is the recognition of the mature portion of the protein by SecB and subsequent binding of the complex by SecA.

Direct interaction between the signal peptide and a component of the Sec-machinery was shown so far only for SecA. They can be chemically cross-linked (Akita *et al.*, 1990) and binding of signal peptide stimulates ATPase activity of SecA (Lill *et al.*, 1990; Miller *et*

al., 1998). The extent of SecA interaction with the signal sequence is directly related to the hydrophobicity of the H domain of the signal peptide (Wang *et al.*, 2000).

The sequences of signal peptides of the various exported proteins are not very well conserved but they possess common structural and physiochemical features (Izard and Kendall, 1994). The length of a typical Sec-pathway signal peptide in *E. coli* ranges from 18 to 30 amino acids. A signal peptide consists of three characteristic domains (Fig. 2.7): a positively charged amino-terminal region, a nonpolar hydrophobic core region and a hydrophilic C-terminal domain containing the leader peptidase cleavage site for the removal of the signal peptide after translocation (Randall and Hardy, 1989; von Heijne, 1990).

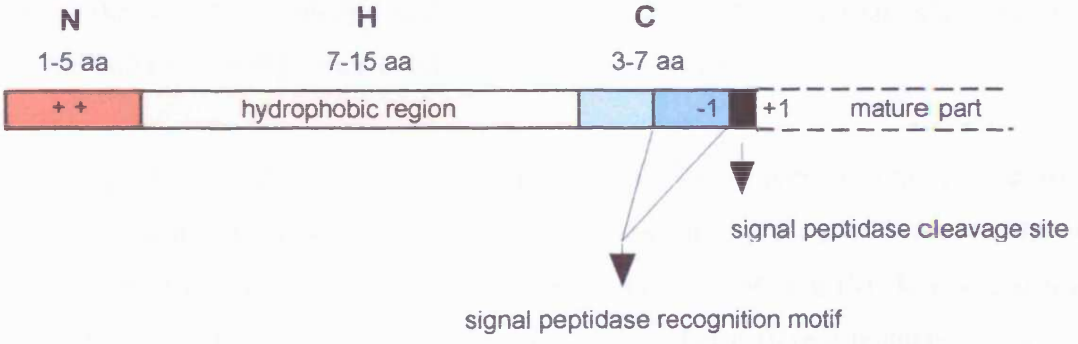


Fig. 2.7 Structure of the signal peptide of secretory proteins with the positively charged N domain (++), the hydrophobic H region and the C domain containing the signal peptidase recognition motif.

The amino acid residues at position –1 and –3 relative to the start of the mature part of the preprotein have small neutral side chains like alanine or glycine (von Heijne, 1984) (Sjostrom *et al.*, 1987). This motif is recognized by type I leader peptidase being responsible for the majority of secretory proteins. A type II leader peptidase cleaves the signal peptides of lipoproteins and recognises a slightly different motif containing a cysteine at position +1 and an amino acid with a large hydrophobic side chain at position –3 (Sankaran and Wu, 1994). The net positive charge of the N region enhances the translocation rate. The positive charges probably facilitate the binding to the negatively

charged surface of the inner membrane (de Vrije *et al.*, 1988; de Vrije *et al.*, 1990). Preproteins with neutral or negatively charged N regions can be processed, but at reduced rates (Gennity *et al.*, 1990). The hydrophobic α-helical core region is the most important part of the signal sequence. Mutations in other domains can be suppressed by increasing the total hydrophobicity of the H domain (Hikita and Mizushima, 1992). A minimum hydrophobicity of this domain is required for secretion (Doud *et al.*, 1993) and translocation rates increase with length and hydrophobicity of the H region (Chou and Kendall, 1990).

The mode of insertion of the signal peptide into the membrane at the translocation site is not fully understood yet. According to the helix reversion hypothesis the signal sequence can form a hairpin like conformation in the cytoplasm due to a helix-breaker residue in the middle of the H region (Shinde *et al.*, 1989). Upon insertion into the lipid bilayer the hairpin-like structure unloops with the help of the transmembrane electrochemical potential and the signal peptide extends into the membrane.

Signal sequences of preproteins dependent on the Tat export system have a similar tripartite structure as signal sequences of Sec-dependent proteins. However, the most obvious difference is the existence of the characteristic S-R-R-x-F-L-K (twin-arginine) motif at the N to H region boundary (Berks, 1996). Other different features of Tat signal sequences are an extended N region of 5 to 25 amino acids and a significantly less hydrophobic H region. The C domain contains basic amino acids that are almost never found in the C region of Sec-signal peptides and might serve as a 'Sec-avoidance' signal (Cristóbal *et al.*, 1999).

A group of autotransporter proteins, like hemoglobin protease secreted by a pathogenic *E. coli* strain also have unusually long signal peptides due to a N-terminal extension of about 25 amino acids, the function of which is unknown (Henderson *et al.*, 1998).

2.2.3 Targeting routes to the Sec-translocon

Preproteins rely on targeting factors early in the route to the membrane. In *E. coli* the two major targeting routes are mediated by the SecB protein or the signal recognition particle (SRP) and its receptor FtsY respectively (Fig. 2.8).

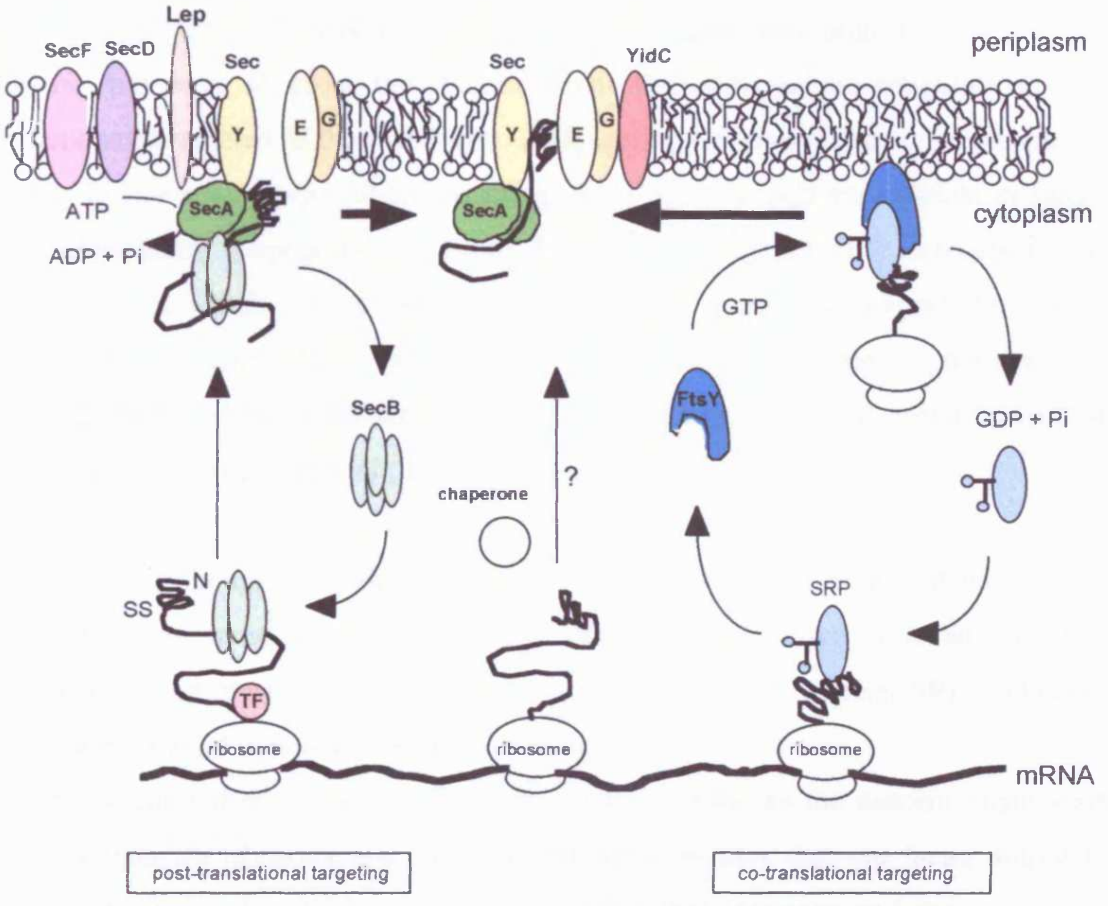


Fig. 2.8 Model of the post- and co-translational targeting routes to the Sec-translocon mediated by SecB/SecA and SRP/FtsY. There may exist some other possibilities of targeting e.g. via other cytoplasmic chaperones. Abbreviations: Lep = leader peptidase; SS = signal sequence.

It is believed that most secretory proteins with cleavable signal sequences are post-translationally directed to the translocon via the cytosolic chaperone SecB (Kumamoto and Francetic, 1993) and the ATPase SecA (Fig. 2.8). SecB binds to the mature part of

the preprotein and keeps it in an unfolded and hence export-competent state. This complex interacts with SecA, which helps the preprotein to cross the inner membrane through the SecYEG channel in an ATP- dependent manner (Danese and Silhavy, 1998; Driessen *et al.*, 1998).

The existence of another targeting pathway was proposed after the identification of cytosolic components that showed strong structural homologies to eukaryotic translocation factors. In eukaryotic cells integral membrane proteins and secretory proteins that have to cross the membrane of the endoplasmic reticulum are co-translationally targeted to the translocon. Early during translation the signal recognition particle recognises a hydrophobic targeting signal of a nascent polypeptide causing a translational arrest (Rapoport *et al.*, 1996). This complex interacts with its receptor in the ER membrane which then allows simultaneous translation and export through the translocation channel. In *E. coli* a similar co-translational targeting pathway was proposed, mediated by the bacterial SRP and its receptor FtsY in co-operation with the SecYE translocon (de Gier *et al.*, 1996; Ulbrandt *et al.*, 1997)

Although it is thought that the SRP- and the Sec-pathway function in parallel and converge at the same translocon, there is conflicting evidence as to whether both pathways overlap in substrate specificity (Kim *et al.*, 2001) or whether SRP exclusively translocates integral membrane proteins (Beha *et al.*, 2003).

The choice for either pathway is probably made as soon as the nascent chain starts emerging from the ribosome and might be dependent on more than one factor. Important features are the hydrophobicity of the exposed signal sequence and the preferential binding affinities of SRP (Valent *et al.*, 1997) and probably trigger factor (Beck *et al.*, 2000) a ribosome-associated chaperone. Several studies showed that the targeting pathway is dictated by the hydrophobicity of the signal peptide. *In vitro* crosslinking experiments have shown that the more hydrophobic the sequence of a signal peptide is, the more efficiently it is bound by SRP. Increasing of the net hydrophobicity of MBP and OmpA signal sequences, two proteins that are normally targeted by SecB, rerouted both proteins into the SRP pathway (Lee and Bernstein, 2001). It was suggested that a threshold level of hydrophobicity is required for substrates of the SRP pathway. With the

recent solution of the crystal structure of the SRP the possibility arose that the RNA subunit of SRP might also play a role in substrate recognition (Batey *et al.*, 2000). It was shown that salt bridges between the SRP-RNA and basic amino acids of the N domain of signal peptides facilitated the binding of SRP despite a less hydrophobic core region (Peterson *et al.*, 2003). This interaction might also provide an explanation for the observation that the extracellular autotransporter protein hemoglobin protease (Hbp) is a substrate for SRP despite a moderately hydrophobic H region, as the Hbp signal peptide contains a more basic N region than usual signal peptides of the general secretion pathway (Sijbrandi *et al.*, 2003). Another factor that has an effect on the targeting pathway of protein translocation is trigger factor (TF), a ribosome-associated chaperone. *In vitro* crosslinking data suggested that *E. coli* SRP fails to recognise signal peptides because trigger factor binds to a N-terminal sequence within the first 125 amino acids of the nascent chain (Beck *et al.*, 2000). Thus, binding of TF would prevent recognition by SRP and route the preprotein into the Sec-pathway. However, on the other hand trigger factor has no affinity for hydrophobic sequences (Valent *et al.*, 1995). Therefore, a nascent hydrophobic sequence might be recognised by SRP before TF can bind to the first amino acids of the mature portion of the protein.

Slow folding of the secretory preprotein was also thought to indirectly direct the preprotein into the SecB dependent targeting pathway. The slow folding might be a result of the presence of signal peptide or alternatively, Lee and Bernstein observed that TF remained bound to cytosolic proteins and preproteins for a relatively extended time period during translation, thus rendering the preprotein dependent on the chaperone SecB (Lee and Bernstein, 2002). They found a significant dependency on SecB only in cells that contained TF.

Unknown sequences in the mature part of the preprotein might however be necessary for promoting SecB binding as a slow folding mutant of a cytoplasmic protein was not exported even in a *prl* strain (Mallik *et al.*, 2002).

2.2.3.1 Components involved in the targeting process

2.2.3.1.1 Trigger factor

Trigger factor (TF) was first identified in *E. coli* in the course of searching for cytosolic components involved in protein export (Crooke and Wickner, 1987). It is a 48 kDa protein (Guthrie and Wickner, 1990) but migrates on SDS-PAGE with a molecular mass of 58 kDa (Hesterkamp *et al.*, 1996). Trigger factor is soluble in the cytoplasm and can bind to the large subunit of ribosomes (Crooke *et al.*, 1988; Lill *et al.*, 1988). The ribosomal protein L23, which is in close proximity to the tunnel exit, was identified as the docking site on the 50S subunit (Kramer, *et al.*, 2002). For ribosome-association the N-terminal 118 amino acids of trigger factor are necessary and sufficient (Hesterkamp *et al.*, 1997). In the last years the functions of trigger factor have been closer investigated.

The central portion of trigger factor shows peptidyl-prolyl *cis/trans* isomerase function (Stoller *et al.*, 1995), which is thought to be important for protein folding. Indeed, trigger factor reveals a high folding activity due to its tight binding to its substrate, which is reflected in a low K_m value of 0.7 μM (Scholz *et al.*, 1997).

Trigger factor has a low affinity for peptides in solution, but shows a broad binding specifity to nascent chains when associated with the ribosome (Patzelt *et al.*, 2001).

Trigger factor can be cross-linked to nascent polypeptide chains of cytoplasmic and secretory proteins (Valent *et al.*, 1995; Hesterkamp *et al.*, 1996). It was suggested however, that trigger factor has a far lower affinity for nascent chains of polytopic membrane proteins enabling the signal recognition particle to select its substrates for co-translational targeting (Beck *et al.*, 2000). It was shown that trigger factor binds preferentially to basic and aromatic residues, probably to protect nascent polypeptides enriched in basic and aromatic amino acids against unproductive interactions with the ribosomal RNA (Patzelt *et al.*, 2001). Surprisingly, binding to unfolded proteins was independent of proline residues and involved the same binding pocket as the PPIase activity (Patzelt *et al.*, 2001).

In its efficient binding to unfolded proteins trigger factor resembles a chaperone (Scholz *et al.*, 1997). A chaperone-like function was confirmed by the finding that simultaneous deletion of the genes of trigger factor and the cytosolic chaperone DnaK is lethal for the

cell, suggesting cooperating functions of the two proteins (Deuerling *et al.*, 1999). In contrast, deletion of either of the genes encoding TF or DnaK has no effect on protein folding or viability under normal growth conditions (Deuerling *et al.*, 1999). The observation that TF can be crosslinked to nascent chains as short as 57 amino acids led to the suggestion that trigger factor is the first chaperone to interact with a newly synthesised protein (Hesterkamp *et al.*, 1996).

2.2.3.1.2 The export-dedicated chaperone SecB

The highly acidic cytoplasmic protein SecB forms a functional homotetramer with subunits of about 17 kDa (Kumamoto, 1989; Watanabe and Blobel, 1989). Homologues of SecB have been found so far only in gram-negative bacteria (Lai and Baumann, 1992). SecB is involved in the translocation process via its function as a chaperone and as a targeting factor. It binds to unfolded forms of precursor proteins maintaining them in a protease-hypersensitive and export-competent state by preventing their folding and aggregation in the cytoplasm prior to secretion (Lecker *et al.*, 1990). It also facilitates the targeting to the translocase by its affinity for SecA (Hartl *et al.*, 1990).

In vitro SecB is able to interact with a broad variety of non-native proteins (Fekkes *et al.*, 1995) but *in vivo* SecB is highly selective (Kumamoto and Francetic, 1993). Only a subset of precursor proteins are affected in their translocation by the deletion of SecB (Kumamoto and Beckwith, 1983; Kumamoto and Beckwith, 1985). It is essential e.g. for the maltose-binding protein (MBP), OmpA and LamB (Kumamoto, 1989) whereas a *secB*-null mutation does not affect the export of PhoA, β-lactamase and the ribose-binding protein (Collier *et al.*, 1990). SecB is also involved in the secretion of some extracellular proteins that are exported via special ABC-transporters in the cytoplasmic membrane (Delepelaire and Wandersman, 1998).

The mechanism of how SecB selects its substrates is poorly understood. The observation that the presence of a signal peptide retards the folding of preproteins (Park *et al.*, 1988) led to the suggestion that this slowing process would allow SecB to bind to newly synthesised precursors (Hardy and Randall, 1991). However, there is some evidence against this model. First, the binding of SecB to substrates is much faster than folding of

newly synthesised polypeptides (Fekkes *et al.*, 1995). Second, the folding rate is determined by the rate of chain elongation and SecB does not only bind to the fully translated polypeptide but also to nascent chains provided a chain-length of more than 150 residues (Kumamoto and Francetic, 1993; Randall *et al.*, 1997). Third, precursors that lack a signal peptide are not slowed in folding but become totally dependent on SecB (Derman *et al.*, 1993). Therefore, the substrate specificity of SecB might rather be due to the recognition of certain binding domains in the precursor than its folding rate (Fekkes *et al.*, 1995).

It is generally believed that SecB binds to multiple sites covering large regions of the mature part of the preprotein (Gannon *et al.*, 1989; Topping and Randall, 1994). Recently binding motifs for SecB were suggested which contain aromatic and basic residues (Kim and Kendall, 1998; Knoblauch *et al.*, 1999). Knoblauch and coworkers identified a motif of about 9 residues which occurs statistically every 20-30 amino acids. The occurrence and affinity of these regions are similar in SecB-dependent and SecB–independent as well as in cytosolic proteins. These results show that SecB could act as a general chaperone but is translocation-dedicated by its interaction with the translocase component SecA.

By investigating several mutations that disrupt the SecB-preprotein interaction a binding site for precursors on SecB was identified as a short hydrophobic polypeptide stretch. It was suggested that the interaction occurs at a ββ-interface (Kimsey *et al.*, 1995) and the major SecB substrates indeed have a high β-sheet content (MacIntyre *et al.*, 1991).

There is some evidence that the SecA-binding site on SecB overlaps the preprotein-binding site in an alternating fashion. In a β-structural conformation (Fasman *et al.*, 1995) the hydrophobic preprotein-binding site and the negatively charged SecA-binding site would be located opposite to each other (Fekkes and Driessen, 1999). Upon binding of SecB to SecA the preprotein is released from SecB and transferred to SecA involving the recognition of the signal peptide by SecA (Fekkes *et al.*, 1998). SecA might lower the affinity of SecB for the preprotein by altering the conformation of the preprotein-binding site on the opposite surface of the β-sheet. In his model SecB would be unable to bind a new preprotein until it is released from SecA at the initiation of the translocation (Fekkes *et al.*, 1997; Fekkes *et al.*, 1998).

SecB is believed to be essential only under conditions of high translocation rates, e.g. during growth in rich medium (Kumamoto and Beckwith, 1985). However, the inability of SecB-null strains to grow in rich medium could be accounted for by the lack of an enzyme involved in phospholipid synthesis due to its overlapping coding region with the termination codon of SecB (Shimizu *et al.*, 1997). Recently it was found that SecB plays an essential role in export only in cells that contained trigger factor (Lee and Bernstein, 2002). The authors concluded that SecB possibly has evolved in bacteria to protect nascent polypeptide chains that reached a significant length after dissociation from trigger factor.

It is not certain that general heat shock proteins are able to substitute for the SecB function in protein translocation. In growth restoration assays of SecB-null mutants it was shown that the heat shock proteins DnaJ and DnaK could support secretion of SecB-dependent substrates (Altman *et al.*, 1991; Wild *et al.*, 1992). On the other hand, chaperones like GroEL and trigger factor can keep a preprotein in an export-competent state but fail to stimulate translocation because they lack an affinity for the components of the translocase (Lecker *et al.*, 1989).

SecB-independent substrates like alkaline phosphatase or β-lactamase seem to rely on general chaperones like GroES/GroEL and DnaK (Kusukawa *et al.*, 1989; Wild *et al.*, 1996; Beha *et al.*, 2003). It was shown that their translocation is disturbed in cells lacking those chaperones. These substrates depend on the signal sequence as a targeting factor. Lowering the level of general chaperones but not of other *sec* genes resulted in an increase of SecB synthesis (Müller, 1996). Recently, it has been suggested that also the Tat pathway might be involved in the translocation of a SecB-independent substrate (Pradel *et al.*, 2003).

Taken together it seems that SecB has the potential to play a role as a general chaperone but differs from other chaperones in its ability to interact with SecA.

2.2.3.1.3 The signal recognition particle and its receptor FtsY

In eukaryotic cells exported proteins are co-translationally targeted to the membrane of the endoplasmic reticulum (ER) by the signal recognition particle (SRP) and its receptor

(SR). The eukaryotic SRP is a ribonucleoprotein comprised of six polypeptides and a 7S RNA (Walter and Blobel, 1982; Luirink and Dobberstein, 1994). Only the 54 kDa subunit binds to the signal sequence (Kurzchalia *et al.*, 1986) and contains a GTP-binding domain (Miller *et al.*, 1993). The binding of the SRP causes arrest of the translational elongation of the nascent chain. The ribosome-bound nascent chain (RNC) in complex with SRP is then targeted to the membrane-associated subunit SRα of the SRP receptor (Walter and Johnson, 1994). Both subunits (SRα and SRβ) of this receptor are GTP-binding proteins and SRβ is an integral membrane protein (Tajima *et al.*, 1986). Upon docking with SRα the RNC is released and export proceeds co-translationally through the translocation channel (Walter and Johnson, 1994).

In *E. coli* a smaller SRP has been discovered. It consists of a 4.5S RNA and a 48 kDa GTPase termed P48 or Ffh (for ***f****ifty* ***f****our* ***h****omologue*) which are the counterparts of the eukaryotic 7S RNA and the 54 kDa subunit of SRP (Poritz *et al.*, 1988; Struck *et al.*, 1988; Bernstein *et al.*, 1989; Romisch *et al.*, 1989). In addition, a receptor for the bacterial SRP, FtsY, was found by its sequence similarity to SRα (Bernstein *et al.*, 1989). In contrast to the eukaryotic SRα, FtsY is not only found membrane-associated but also soluble in the cytoplasm. However, the function of the cytosolic FtsY remains unclear because *E. coli* is able to grow without the soluble form of FtsY (Zelazny *et al.*, 1997).

The eukaryotic SRα is co-translationally targeted to the ER membrane and anchored via the SRβ subunit (Young *et al.*, 1995). There is some evidence that *E. coli* FtsY is also directed to the membrane during its translation (Herskovits *et al.*, 2000). It was proposed that this mode of targeting is essential for FtsY activity (Herskovits *et al.*, 2001).

FtsY contains two major domains (de Leeuw *et al.*, 1997). The N-terminal acidic A-domain is probably necessary to co-translationally direct the C-terminal catalytic NG-domain to the membrane (Zelazny *et al.*, 1997). The mechanism of FtsY association with the membrane is unclear. FtsY contains no hydrophobic helices and no SRβ homologue has been identified so far. However, recently it was shown that FtsY interacts directly with anionic phospholipids, and thus FtsY might associate with the membrane via a direct protein-lipid interaction (de Leeuw *et al.*, 2000).

Despite several similarities to the eukaryotic system the role of the bacterial components in targeting and translocation are not yet clearly defined. FtsY interacts with SRP *in vitro*

in a GTP-dependent manner (Miller *et al.*, 1994) and it has been demonstrated that both bacterial components can replace their mammalian counterparts in a mammalian *in vitro* translocation system (Powers and Walter, 1997). The 4.5S RNA, Ffh and FtsY are essential for the viability of the cell (Brown and Fournier, 1984; Phillips and Silhavy, 1992; Seluanov and Bibi, 1997) but their depletion has a rather mild effect on the export of only a subset of secretory preproteins, with pre-β-lactamase showing the strongest accumulation in the cytoplasm (Poritz, 1990; Ribes, 1990; Luirink, 1994). However, it has been argued that the significance of these defects is uncertain and maybe an indirect result as targeting of SecY turned out to be SRP dependent. Furthermore, it was shown that 4.5S RNA also plays a role in translation and its depletion causes dramatic reduction in protein synthesis (Jensen *et al.*, 1994).

Valent and coworkers demonstrated that Ffh binds preferentially to nascent, ribosome-associated polypeptides which carry particularly hydrophobic targeting signals (Valent *et al.*, 1995). Integral cytoplasmic membrane proteins are synthesised with hydrophobic signal anchor sequences and it was shown that the biogenesis of several inner membrane proteins is dependent on SRP and FtsY *in vivo*. These findings strongly suggest that both components function predominantly in the targeting of inner membrane proteins (MacFarlane and Müller, 1995; de Gier *et al.*, 1996; Seluanov and Bibi, 1997; Ulbrandt *et al.*, 1997; Herskovits *et al.*, 2000). Based on theses data and the results of several cross-linking experiments a model for the SRP-dependent targeting in *E. coli* was proposed which resembles the eukaryotic co-translational targeting mechanism (Valent *et al.*, 1998). According to this model hydrophobic ribosome-bound targeting signals are bound by the SPR and this complex is recognised by cytoplasmic or membrane-bound FtsY (Fig. 2.8). Upon association of FtsY with the membrane the ribosome together with the nascent chain is released after binding of GTP to FtsY and SRP. The preprotein inserts into the membrane at the translocase or via a different mechanism. SRP is released by GTP-hydrolysation and can enter the cycle again (Valent *et al.*, 1998; Fekkes and Driessen, 1999). However, recent results from Herskovits and Bibi could not confirm the targeting role of the SRP *in vivo* (Herskovits and Bibi, 2000). Their experiments suggest that Ffh may rather play a role in releasing the ribosome from the membrane and transferring it to the translocase.

2.2.4 Membrane insertion of integral membrane proteins

2.2.4.1 Structure and Topology of integral membrane proteins

Membrane proteins exist in a variety of shapes and sizes but share common basic features. The transmembrane domains form α-helical secondary structures of about 20 amino acids with largely hydrophobic side chains. Bitopic membrane proteins consist of one α-helix connecting two domains on either side of the membrane whereas polytopic membrane proteins possess several membrane-spanning segments, which are connected by hydrophilic cytoplasmic and periplasmic loops.

The topology, that is the number and orientation of transmembrane domains can be predicted by analysing the amino acid sequence for hydrophobic properties and by following the positive inside rule (von Heijne, 1992; Traxler *et al.*, 1993). This rule states that cytoplasmic domains contain an excess of positively charged residues like arginine and lysine (von Heijne, 1986) whereas periplasmic loops have a negative or no net charge. This rule however does not apply for periplasmic loops of more than 60 amino acids, as there is no reduction in the content of Arg or Lys residues (Andersson and von Heijne, 1993). It was suggested that the length of the periplasmic domain determines the mechanism of its translocation (see 2.2.4.2.)(von Heijne, 1986).

A variety of biochemical and molecular genetic techniques can be used to verify the predicted membrane topology. One possibility is to investigate the access of specific target sites in the periplasmic portions of the protein by reagents which are not able to penetrate the membrane. Such reagents could be certain chemicals, proteases or antibodies. An example for a specific target site introduced in the sequence of an outer membrane protein is the cleavage site of the TEV-protease derived from tobacco etch virus (Mondigler, 1997). Another possibility to study membrane topology is the use of sandwich fusions. In this approach reporter-proteins are inserted into the membrane proteins. The enzymatic activity of the reporter can be used to determine the localisation of the fusion junction (Boyd *et al.*, 1987; Manoil *et al.*, 1990).

2.2.4.2 *Mechanism and Sec- dependency of membrane insertion*

A simple mechanism for the assembly of membrane proteins is the sequential insertion of hydrophobic segments as they emerge from the ribosome with alternating orientation of each adjacent domain (Blobel, 1980; Friedlander and Blobel, 1985). A signal anchor segment is orientated with its N-terminus in the cytoplasm and the C-terminus out in the lumen or the periplasm. It is followed by a stop transfer segment with the opposite orientation.

Several observations in *E. coli* however, strongly suggest a more complicated mechanism involving the coordination of multiple topogenic signals distributed over the nascent chain (Ehrmann and Beckwith, 1991; Gafvelin and von Heijne, 1994; Gafvelin *et al.*, 1997; van Geest *et al.*, 1999). In addition, recent studies implicate that distinct proteinaceous factors are involved in the assembly of integral membrane proteins (IMPs). There is strong evidence that many membrane proteins depend on the SRP and FtsY for proper insertion (MacFarlane and Müller, 1995; de Gier *et al.*, 1996). This would suggest a co-translational mode of insertion similar to the eukaryotic system, where the Sec61 translocon provides a channel that opens laterally and allows the exit of transmembrane segments into the lipid bilayer of the ER membrane (Mothes *et al.*, 1997).

The involvement of the bacterial SecYEG channel in such a scenario would be very likely and there is some experimental evidence to support this suggestion (Traxler and Murphy, 1996; Newitt and Bernstein, 1998).

Recently, a 60 kDa integral membrane protein called YidC was identified as an essential component of membrane insertion either in conjunction with the Sec-translocase or without (Samuelson *et al.*, 2000; van der Laan *et al.*, 2001). So far, all IMPs investigated were found to be dependent on YidC (Chen *et al.*, 2002), even some IMPs that were previously thought to insert spontaneously into the membrane (Samuelson *et al.*, 2001). YidC is a polytopic membrane protein with six transmembrane domains (Sääf *et al.*, 1998). It is a homologue of the mitochondrial Oxa1p and the thylakoidal Alb3 proteins that are involved in membrane protein assembly in mitochondria or chloroplasts respectively (Luirink *et al.*, 2001). A proposed function of YidC associated with the Sec-translocon is the release of the transmembrane segment of the IMP from the channel into the lipid bilayer (Scotti *et al.*, 2000; Urbanus *et al.*, 2001).

Membrane insertion was shown to occur dependently or independently of the components of the Sec-translocase. It is possible that different membrane proteins may exclusively use one or the other of those insertion mechanisms (Lee *et al.*, 1992). Alternatively there might not be a clear-cut distinction between the two mechanisms, as different domains of the same proteins might require different components. The degree of Sec-dependency is difficult to determine because a certain protein could use all or only some components of the Sec-translocase. In the case of SecA it is possible that the degree of its requirement is a function of the number of ATP-binding/hydrolysis cycles, which are necessary for translocation; or a protein only uses SecA as a targeting factor to the SecYEG channel without dependency on translocation ATPase (Andersson and von Heijne, 1993; von Heijne, 1994). Hydrophilic periplasmic loops are the most likely domains of inner membrane proteins to be dependent on Sec-components. It is thought that the length (Andersson and von Heijne, 1993) and the number of positively charged residues (Andersson and von Heijne, 1994) are determinants for the degree of Sec-dependency in a way that domains larger than 60 residues would become Sec-dependent. This suggestion was experimentally supported but also questioned (Kuhn, 1988; Andersson and von Heijne, 1993; Whitley *et al.*, 1994). Other determinants for Sec-dependency were shown to be located in the surrounding sequences of translocated segments (von Heijne, 1994; Sääf *et al.*, 1995).

The role of SecA in the SRP-mediated targeting pathway remains especially unclear and experimental data for diverse SRP-substrates are contradictive (Qi and Bernstein, 1999; Scotti *et al.*, 1999). For example, the integral membrane protein MalF possesses a large second periplasmic loop of about 180 amino acids which was reported to insert independently of SecA (McGovern and Beckwith, 1991) as well as SecA-dependent (Traxler and Murphy, 1996).

In a very recent study it was shown that *E. coli* possesses a second mechanism of membrane insertion that is fundamentally distinct from that employed by other IMPs (Hatzixanthis *et al.*, 2003). The authors showed that at least five Tat-substrates containing hydrophobic C-terminal transmembrane helices were anchored in the inner membrane via the Tat system without requirement of YidC.

2.3 NIa proteinase of tobacco etch virus (TEV protease)

Tobacco etch virus (TEV) is a member of the potato virus Y family (*Potyviridae*) which belongs to the picornavirus superfamily (Koonin and Dolja, 1993). It infects the tobacco plant *Nicotiana tabacum* and causes necrosis of leafs (Hollings, 1981). Potyviruses have a flexuous rod shape and possess a single-stranded RNA genome that is polyadenylated at the 3' end (Hari *et al.*, 1979) and covalently linked to a virus-encoded protein (VPg) at the 5' end (Riechmann *et al.*, 1992). The TEV genome of about 9.5 kb is translated into a single polyprotein of 351 kDa (Allison *et al.*, 1986; Dougherty and Semler, 1993). Three viral proteinases catalyse the co- and post-translational processing into intermediate precursors and at least eight mature proteins (Carrington *et al.*, 1990; Dougherty and Semler, 1993)(Fig. 2.9).

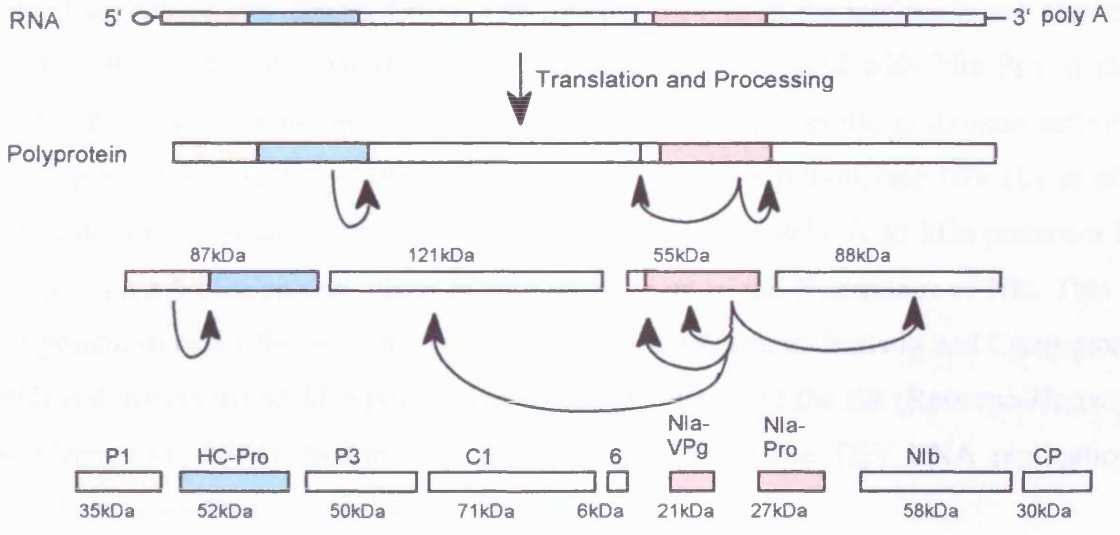


Fig. 2.9 RNA genome of tobacco etch virus and model of polyprotein processing. The VPg protein linked to the 5' terminus of the RNA is represented as an open circle. Arrows indicate points of cleavage.

The two proteinases P1 and HC-Pro are localised in a N-terminal 87 kDa segment of the polyprotein and are responsible for two autocatalytic cleavage events (Carrington *et al.*,

1989). The remaining processing reactions are catalysed by the "nuclear inclusion a" protein (NIa proteinase, also termed TEV protease) (Carrington and Dougherty, 1987).
Apart from playing a key role in polyprotein processing each proteinase also has other functions during the infection process (Cronin *et al.*, 1995; Verchot and Carrington, 1995; Daros and Carrington, 1997). The multiple functions of NIa are associated with distinct domains and subcellular localisation of the protein (Carrington *et al.*, 1991; Dougherty and Parks, 1991). The 21 kDa N-terminal VPg domain attaches covalently to the 5' terminus of viral RNA, either independently or as a part of full length NIa and most likely plays a role in the initiation of RNA synthesis (Murphy *et al.*, 1990; Daros and Carrington, 1997)
The proteolytic activity is located in the C-terminal 27kDa domain, termed NIa-Pro (Carrington and Dougherty, 1987; Dougherty and Parks, 1991). NIa contains a nuclear localisation signal (Restrepo *et al.*, 1990) and its 49 kDa form aggregates into a nuclear inclusion body in the infected cell together with the NIb and P3 proteins (Knuhtsen *et al.*, 1974; Langenberg and Zhang, 1997). The function of NIa in the nucleus is not known. On the other hand, at least three functions can be associated with NIa-Pro in the cytoplasm or in a membrane bound complex: a sequence specific proteinase activity (Carrington and Dougherty, 1987), binding of TEV RNA-polymerase NIb (Li *et al.*, 1997) and a RNA-binding activity (Daros and Carrington, 1997). A 55 kDa precursor is formed with a 6 kDa protein which is located adjacent to the N-terminus of NIa. This 6 kDa protein impedes the nuclear translocation of NIa (Restrepo-Hartwig and Carrington, 1992) and directs the 55 kDa polyprotein to the membrane of the ER (Restrepo-Hartwig and Carrington, 1994). In this way it probably anchors the TEV RNA replication apparatus to membranous sites (Schaad *et al.*, 1997).
NIa-Pro catalyses cleavage of a consensus motif of seven amino acids (Fig. 2.10) with strong conserved residues at positions P6 (glutamate), P3 (tyrosine), P1 (glutamine) and P'1 (serine or glycine). The amino acid at P4 typically has a small neutral side chain, and residues at position P2 and P5 may have a regulatory function by modulating cleavage rate (Dougherty *et al.*, 1989). Cleavage occurs between the dipeptides glutamine-serine or glutamine-glycine (Carrington and Dougherty, 1987).

NIa proteinase shows structural similarities with cellular serine proteases, however, with the substitution of a cysteine-residue in the active site (Carrington and Dougherty, 1987; Bazan and Fletterick, 1988). The catalytic triad is formed by His234, Asp269 and Cys339 of the 49 kDa protein (Dougherty *et al.*, 1989). Proteolytic activity is reduced to 1-2% of wild-type activity when cysteine is replaced by serine and can be inhibited with zinc ions and iodacetamide. Most other serine or cysteine proteinase inhibitors show only minimal or no effects (Dougherty and Semler, 1993).

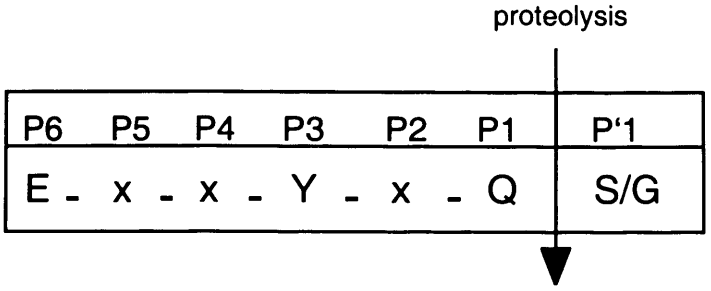


Fig. 2.10 Heptapeptide consensus sequence necessary for TEV protease recognition and cleavage.

Several features make NIa proteinase (from hereon called TEV protease) a suitable tool for many different molecular biological studies *in vivo* and *in vitro*. First, the protease is highly substrate specific due to its conserved heptapeptide recognition sequence. Second, this recognition sequence can be introduced into proteins that are not natural substrates of TEV protease. Thus, they can be transformed into a target for TEV protease (Carrington and Dougherty, 1988). Third, expression of TEV protease in *E. coli* cytoplasm has no negative effect on cell viability.

TEV protease can be used to study the topology of membrane proteins (Mondigler, 1997; Faber *et al.*, 2001), to cleave- off affinity tags in protein purification (Parks *et al.*, 1994) or to process fusion proteins or domains in the cytoplasm (Kapust and Waugh, 2000; Herskovits *et al.*, 2001). Another possible application is the inactivation of essential proteins *in vivo* (Mondigler and Ehrmann, 1996).

2.4 Aims of this study

2.4.1 Aims concerning structure and function of SecA

SecA is one of the central components of the *E. coli* translocase mediating post-translational export of precursor proteins through the SecYEG channel. Among its various activities it functions as a receptor for the SecB-preprotein complex (Hartl *et al.*, 1990) and supplies energy for the translocation process by ATP hydrolysis (Lill *et al.*, 1989). However, a role of SecA in co-translational protein translocation, i.e. targeting and insertion of inner membrane proteins remains elusive. For instance, there is conflicting evidence concerning the SecA-dependency of the assembly of the complex inner membrane protein MalF (McGovern and Beckwith, 1991; Traxler and Murphy, 1996). Such controversial results are probably due to varyingly sensitive experimental procedures or differences in the intensity of SecA inhibition. It cannot be ruled out that some proteins may have a high affinity to SecA and require very low levels of SecA activity for translocation. Thus, strong inhibition of its function or exhaustive depletion of the functional protein might be necessary to observe effects on membrane insertion. SecA is a dimer in solution (Akita *et al.*, 1991) and has interaction sites for several binding partners. However, its three-dimensional structure in solution and especially within the membrane is not completely clear yet. Therefore, in this study we sought to:

a) Improve a method of site-specific proteolysis to completely inactivate functional SecA by TEV protease. The TEV protease cleavage sequence had been previously introduced into the *secA* sequence at a few distinct positions for specific *in vivo* proteolysis and it had turned out that cleavage efficiency was dependent on the position within the SecA protein (Mondigler and Ehrmann, 1996).
b) Investigate the supposed role of SecA in inner membrane protein insertion using a strain where SecA could be completely inactivated and a sensitive assay to detect translocation defects via cytoplasmic biotinylation of a PSBT domain (Jander *et al.*, 1996).

c) Introduce the TEV protease cleavage sequence into SecA at numerous separate positions for further characterisation of the resulting SecA-constructs. This approach could be useful to investigate SecA structure via TEV protease accessibility tests as effective proteolysis gives evidence of the surface exposure of the respective cleavage site.

d) Gain hints on cellular factors that might be involved in protein translocation by using DNA macroarray technique.

2.4.2 Aims concerning function of Ffh and FtsY

Ffh, the protein component of the bacterial SRP, and its receptor FtsY are supposed to play a key role in targeting of membrane proteins to the inner membrane (MacFarlane and Müller, 1995; Seluanov and Bibi, 1997). Both proteins are essential for cell viability (Phillips and Silhavy, 1992; Luirink *et al.*, 1994). However, it is not entirely clear if the targeting function is responsible for this essentiality. Not all inner membrane proteins seem to rely on SRP mediated targeting (Ulbrandt *et al.*, 1997) and experiments with SRP-deficient yeast cells indicated that SRP targeting function could be at least partially by-passed (Hann and Walter, 1991). In addition, previous studies indicated a role of cellular chaperones in the targeting of soluble proteins (Wild *et al.*, 1992; Wild *et al.*, 1996). Therefore, chaperones might also support the co-translational targeting pathway. Our plans in this study were to:

a) Confirm the supposed function of Ffh and FtsY in inner membrane assembly by using Ffh- and FtsY depletion strains and biotinylation assays as mentioned above, and use the results as a control for the SecA-inactivation assays.

b) Gain hints for possible alternative targeting routes, putative interaction partners or additional functions of Ffh and FtsY using DNA macroarray technique.

3 Material and Methods

3.1 Enzymes and antibodies

All restriction enzymes used for cloning were ordered from New England Biolabs (Beverly, MA, USA). Other enzymes and proteins were ordered from the suppliers listed in Table 3.1 below.

Enzymes and proteins	**Supplier**
AMV reverse transcriptase	Sigma Genosys
Calf intestine alkaline phosphatase	Boehringer Mannheim (Lewes, East Sussex, UK)
Klenow fragment	New England BioLabs
Lysozyme	Sigma (Poole, Dorset, UK)
M-MLV reverse transcriptase	Promega
Shrimp alakaline phosphatase (SAP)	Roche (Lewes, East Sussex, UK)
Streptavidin alkaline phosphatase conjugate	Amersham Pharmacia Biotech (Little Chalfont, UK)
T4 DNA Ligase (low and high concentration))	Promega (Madison, WI, USA)
Taq *DNA polymerase*	Promega
Pfu *DNA polymerase*	Stratagene (Cambridge, UK)
BSA	Sigma (Poole, Dorset, UK)

Tab. 3.1 Enzymes and proteins and their suppliers

All antibodies used (Table 3.2) are part of the laboratory collection except the secondary antibody. Anti-Rabbit IgG alkaline phosphatase conjugated antibody was ordered from Sigma (Poole, Dorset, UK).

Antibodies	Molecular mass [kDa]
α-AP	49.4
α-DegP	49.0
α-DnaK	68.9
α-Ffh	49.6
α-FtsY	54.3
α-GroEL	57.2
α-HtpG	71.2
α- IbpB	16.0
α-MalF	57.0
α-SecA	102.0
α-TEV protease	49.0
α-Trigger factor	48.0

Tab. 3.2 Antibodies and molecular mass of the respective proteins.

3.2 Chemicals and other materials

Chemicals and laboratory equipment not stated in Table 3.3 below were ordered from DIFCO, Greiner Labortechnik (Darmstadt, Germany) and Merck (Dorset, UK)

Chemicals / Materials	Supplier
Acrylamide	ICN (Basingstoke, UK)
Ampicillin	AppliChem (Darmstadt, Germany))
APS	Sigma (Poole, Dorset, UK)
Big Bye terminator cycle sequencingV2.0 kit	Applied Biosystems, ABI
Chloramphenicol	Sigma
DMS	Sigma
DMSO	AppliChem (Darmstadt, Germany)
DNA standard-λ-DNA *BstEII* digest	New England Biolabs (Beverly, MA, USA)

Easytides 33P-deoxycytidine	New England Nuclear
E. coli labelling primers	Sigma Genosys
Ficoll	Sigma (Dorset, UK)
Hybridisation kit	Sigma Genosys
Kanamycin	AppliChem (Darmstadt, Germany)
Micro Bio-Spin ®6 Chromatography columns	Bio-Rad
NBT	AppliChem (Darmstadt, Germany)
Oligonucleotide-Primer	MWG (Ebersberg, Germany)
Panorama *E. coli* gene arrays	Sigma Genosys
Polyvinylpyrrolidone	Sigma
Protein standards (Precision protein standard, prestained protein marker, broad range, low range)	Bio-Rad, New England Biolabs
QIAprep Spin Miniprep kit	QIAGEN (Crawley, West Sussex, UK)
QIAquick gel extraction kit	QIAGEN (Crawley, West Sussex, UK)
QIAquick PCR purification kit	QIAGEN (Crawley, West Sussex, UK)
RNAprotect bacteria Reagent	QIAGEN (Crawley, West Sussex, UK)
RNase-free DNase Set	QIAGEN (Crawley, West Sussex, UK)
RNeasy mini kit	QIAGEN (Crawley, West Sussex, UK)
TEMED	Genaxis, France
Tetracycline	Fluka
Salmon testes DNA	Sigma
Saran food wrap	Fisher scientific (Leicester, UK)
Sealkem agarose	FMC Bioproducts (Rockland, USA)
Sodium dodecyl sulfate	ICN Biomedicals Inc.
Spectinomycin	Sigma
XP (BCIP)	Sigma
X-GLUC	PEQLab (Erlangen, Germany)

Tab. 3.3 Chemicals and materials and their suppliers

3.3 Bacterial strains, Plasmids and Bacteriophages

All bacterial strains used and/or constructed in this study are derivatives of *Escherichia coli* K-12. The strains are listed in Table 3.4 below, and Table 3.5 summarises all plasmids that were constructed and/or used in this study.

Strain	**Relevant genotype**	**Reference**
AFF220	MC4100 *lamB*Δ111 *lon*::Tn*10*	laboratory collection
CC1	MM58 Δ*tig*::*kan*	(Conz, 2000)
CC2	MM63 Δ*tig*::*kan*	(Conz, 2000)
CC3	MM52 Δ*araD*174	(Conz, 2000)
CC4	MC4100Δ*ara* Δ*tig*::*kan*	(Conz, 2000)
DHB3	Δ*malF*3 Δ*phoA* (*PvuII*) *phoR recA*::*cat* *araD*139 Δ(*ara-leu*)7697 Δ*lacX*74 *galE galK thi*	(Boyd *et al.*, 1987)
DHB4	DHB3 F'*lacI*^{q}pro	(Boyd *et al.*, 1987)
DHB6500	SM551 λInChII	D. Boyd
EZ4	*rpsL* Δ(*add-uid-man*)	(Chang *et al.*, 1991)
N4156::pAra14-FtsY'	N4156 *bla araC* P_{araBAD}-*ftsY*	(Luirink, 1994)
KS272	MC1000(F- *araD*139Δ(*ara-leu*)7697 Δ*lacYZI*74 *galE galK rpsL*)	(Strauch and Beckwith, 1988)
MC4100Δ*ara*	F⁻ Δ*araD*174 Δ(*argF-lac*) *rpsL*150 *deoC*1 *relA*1 *ptsF*25 *flbB*5301 *rbsR U*169	laboratory collection
MC4100	F⁻ Δ*araD*139 Δ(*argF-leu*) *rpsL*150 *deo*C1 *relA*1 *ptsF*25 *flbB*5301 *rbsR U*169	(Casadaban, 1976)
MM52	MC4100 *secA*51(Ts)	(Oliver and Beckwith, 1981)
MM58	MC4100 *secA*252	(Mondigler, 1997)
MM63	MC4100 *secA*195	(Mondigler, 1997)
RU105	*clpP*::*cm*, MM312 (*araD*139 *flb*5301 *malT* *ptsF*25 *rbsR relA*1 *rpsL*150 *zjb*-729::Tn*10* Δ(*argF-lac*) U169 Δ*malB*112)	laboratory collection
SM551	F⁻ Δ*lac*(Ms265) *mel nalA supF*58	(Boyd *et al.*, 2000)
TH2	DHB3 *malI*::Tn*10* *treR*::Tn*10* Δ*malF*, Cm^R	this study
TH4	MC4100Δ*ara bla secA*att	this study
TH5	MC4100Δ*ara bla secA830*att	this study
TH6	MC4100Δ*ara bla secA252*att	this study

TH7	MC4100Δ*ara bla secA195*att	this study
TH8	MC4100Δ*ara bla secA195,2*att	this study
TH4K	TH4 Δ*secA*, Kan^R	this study
TH5K	TH5 Δ*secA*, Kan^R	this study
TH6K	TH6 Δ*secA*, Kan^R	this study
TH7K	TH7 Δ*secA*, Kan^R	this study
TH24	MC4100Δ*ara* Δ*secA*, *bla secA*att	this study
TH25	MC4100Δ*ara* Δ*secA*, *bla secA830*att	this study
TH26	MC4100Δ*ara* Δ*secA*, *bla secA252*att	this study
TH27	MC4100Δ*ara* Δ*secA*, *bla secA195*att	this study
TH46	TH26 Δ*tig*::*kan*	this study
TH47	TH27 Δ*tig*::*kan*	this study
WAM 113	MC4100 *ffh*::*kan* att::R6Kori P_{araBAD}-*ffh araC*	(Phillips and Silhavy, 1992)

Tab. 3.4 Bacterial strains. All strains are derivatives of *Escherichia coli* K12.

Plasmid	**Genotype**	**Resistance**	**Reference**
pACYC184	pA15 origin of replication	Tc, Cm	(Chang and Cohen, 1978)
pBAD18-Cm	P_{araBAD} MCS2*, *rrnB*T1T2 *araC* pBR322 ori *bla*::*cm*	Cm	(Guzman *et al.*, 1995)
pBR322	Cloning vector	Amp, Tc	(Bolivar *et al.*, 1977)
pCC6	pMM13 P_{tac} *tig*(1-144)-TEV	Tc	(Conz, 2000)
pCS19	pQE60, *lacI*^{q} ColE1 origin	Amp	C. Spiess
pCP20	λcI857(ts) ts-replicon, Flp yeast recombinase gene	Amp, cm	(Datsenko and Wanner, 2000)
pDHB32	*malF*	Amp	(Boyd *et al.*, 1987)
pEDIE-2	PCS *uidA*	Amp	(Conz, 2000)
pHP42	pBAD18 *ftsQ*-PSBT	Amp	(Tian and Beckwith, 2000)
pKD4	oriRγ, FRT -flanked Kan^r	Amp, Kan	(Datsenko and Wanner, 2000)
pKD46	oriR101, *repA*101ts, araBp-gam-bet-exo, Red recombinase helper plasmid	Amp	(Datsenko and Wanner, 2000)
pKD46spec	pKD46 *bla*::*kan*	Kan	this study
pMF8	pBR322 gene*X* *secA*	Amp	(Schmidt *et al.*, 1988)
pMIC16	P_{malK}-*phoA* (out of reading frame), *lacZ*	Amp	(Braun, 1996)

pMKL18	*secA*	Amp	M. Klose
pMM13	pACYC184, P_{tac}-TEV-GST	Tc	(Mondigler, 1997)
pMM51	*secA*830	Amp	(Mondigler, 1997)
pMM52	*secA*252	Amp	(Mondigler, 1997)
pMM54	*secA*195	Amp	(Mondigler, 1997)
pMM55	*secA*195,2	Amp	(Mondigler, 1997)
pQE60	*bla,* 6x His tag	Amp	Qiagen
pSecA60	pEDIE-2 Δ*uidA* P_{araB} -*secA*60	Amp	(Conz, 2000)
pSecA229	pEDIE-2 Δ*uidA* P_{araB} –*secA*229	Amp	this study
pSecA295	pEDIE-2 Δ*uidA* P_{araB} –*secA*295	Amp	(Conz, 2000)
pSecA296	pEDIE-2 Δ*uidA* P_{araB} –*secA*296	Amp	this study
pSecA518	pEDIE-2 Δ*uidA* P_{araB} –*secA*518	Amp	this study
pSecA471	pEDIE-2 Δ*uidA* P_{araB} –*secA*471	Amp	(Conz, 2000)
pSecA497	pEDIE-2 Δ*uidA* P_{araB} –*secA*497	Amp	(Conz, 2000)
pTH2	pMIC16 P_{malK}-*phoA* shifted in correct reading frame, Δ*lacZ*	Amp	this study
pTH4	*secA*::*kan*	Amp, Kan	this study
pTH9	pZS*2tet0-1, *kan*::*spec*	Spec	this study
pTH10	pTH9, $P_{Ltet0-1}$ *tig*(1-144)-TEV	Spec	this study
pTH12	pSecA229 *secA*229short	Amp	this study
pTH13	pSecA295 *secA*295short	Amp	this study
pTH14	pSecA296 *secA*296short	Amp	this study
pTH15	pSecA518 *secA*518short	Amp	this study
pTH20	pBAD18cm, *ftsQ*-PSBT	Cm	this study
pTH22	pBAD18cm, *malF*66-PSBT	Cm	this study
pTH23	pBAD18cm, *malF*274-PSBT	Cm	this study
pTH24	pBAD18cm, *phoA*-PSBT	Cm	this study
pTH25	pVC19, *ftsQ*-PSBT	Tc	this study
pTH27	pVC19, malF66-PSBT	Tc	this study
pTH28	pVC19, *malF*274-PSBT	Tc	this study
pTH29	pVC19, *phoA*-PSBT	Tc	this study
pTHS19	pVC19 Δ6xhis-tag	Tc	this study
pVC19	pQE60, $lacI^q$ Tc^r pA15 ori,	Tc	V. Coupe
pZS*2tet0-1 TEV +tet rep	$P_{Ltet0-1}$-TEV-GST, *tetR*	Kan	(Lutz and Bujard, 1997) and E. Bibi

Tab. 3.5 Plasmids

Bacteriophages used for strain construction are listed in Table 3.6 below.

Bacteriophage	**Description**	**Reference**
$P1_{vir}$	transducing phage	laboratory collection
λInChI	λ Sam cI857, homologues sequence to pBR derived plasmids	(Boyd *et al.*, 2000)
λInChII	λ Sam cI857, homologues sequence to pUC derived plasmids	(Boyd *et al.*, 2000)

Tab. 3.6 Bacteriophages

3.4 Media

3.4.1 Liquid and solid media

Media for liquid cultures and agar plates were prepared according to Sambrook *et al.* (Sambrook *et al.*, 1989). For liquid cultures the ingredients were dissolved in deionised H_2O to a final volume of 1 litre and autoclaved for 20 minutes. Solid medium for agar plates was prepared accordingly with the addition of 17 g/l Bacto agar (as not otherwise stated) before autoclaving.

LB Medium (Luria- Bertani Medium)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g

NZA Medium

NZ amine A*	10 g
Bacto-yeast extract	5 g
NaCl	7.5 g

* NZ amine: enzymatic Casein hydrolysate (Sigma)

M9 Medium

M9 salts (10x): dissolved in 1 litre dH_2O

$Na_2HPO_4.2H_2O$	76.54 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

This salt solution was autoclaved and added to the medium in 1x concentration.

Further ingredients (final concentration):

$MgSO_4$	1 mM
$CaCl_2$	0.1 mM (from 100 mM stock solution)
Carbon source	0.4%
Thiamine	0.01%
Amino acids	1x (from 100x or 50x stock solutions respectively)

The volume was adjusted to 1 litre with sterile dH_2O.

For agar plates 17 g of Bacto agar were autoclaved in 900 ml dH_2O. Then 100 ml 10x M9 salts and a carbon source were added.

SOB Medium

Bacto-tryptone	20 g
Bacto-yeast extract	5 g
NaCl	0.5 g
250 mM KCl	10 ml

pH 7 was adjusted with 5 M NaOH and the volume was adjusted to 1 litre with dH_2O.

Before use 5 ml of sterile $MgCl_2$ (2 M) were added.

SOC Medium

SOC medium was prepared like SOB medium (see above) but after autoclaving at about 60°C 20 mM sterile glucose was added to the final volume.

TB-topagar

Bacto-tryptone	10 g
NaCl	5 g
Bacto Agar	7 g

3.4.2 Media supplements

Media supplements (Table 3.7) were dissolved in an appropriate solvent. L-arabinose, thiamine, sodium-citrate and X-GLUC were sterilised by filtration through a 0.2 µm filter. The supplements were added from stock solutions to the autoclaved medium.

Supplement	**Solvent**	**Final concentration**
Ampicillin	dH_2O	100–200 µg/ml
Anhydrotetracycline	dH_2O	100 ng/ml
L-arabinose	dH_2O	0.1% -0.2%
Chloramphenicol	70% ethanol	30 µg/ml
IPTG	dH_2O	10–100 µM
Kanamycin	dH_2O	100 µg/ml
Sodium-citrate	dH_2O	20 mM
Spectinomycin	dH_2O	100 µg/ml
Tetracycline	100% ethanol	5 µg/ml
Thiamine	dH_2O	1 µg/ml
X-GLUC	100% DMF	50 µg/ml

Tab. 3.7 Media supplements

The amino acid mix for M9 medium supplementation was prepared according to Carol Kumamoto (pers. com.) as follows and added to the medium in 1x concentration:

Hydrophobic amino acids (Ile,Phe,Tyr,Tyr) 100x:

250 mg of every amino acid were dissolved in 100 ml dH_2O, boiled and adjusted to pH 8.0 with $NaCO_2$ and sterilised by filtration.

Hydrophilic amino acids (except cystein) 50x:

250 mg of every amino acid were dissolved in 50 ml dH_2O and sterilised by filtration.

3.5 Microbiological methods

3.5.1 Sterilisation

Media, buffers and glassware were sterilised by autoclaving for 20 minutes at 121°C and a pressure of 1 bar. Heat sensitive solutions were sterilised by filtration using filters with 0.2 µm pore size.

3.5.2 Growth conditions and storage

Liquid cultures were grown at 28°C or 37°C in a culture roller-drum (ca. 50 rpm). Cultures with a volume of up to 5 ml were grown in 20 ml glass reaction tubes, whereas cultures over 5 ml were grown in Erlenmeyer flasks with a volume 5 times bigger than the culture volume in incubator chests shaking at 220 rpm. For storage 126 µl DMSO were added to 1.7 ml of an overnight culture and frozen at – 80°C. Bacterial colonies on agar plates were grown at 28°C, 37°C or 42°C.

3.5.3 Determination of cell density

The density of a culture was determined as the optical density at a wavelength of 578 nm or 600 nm with a photometer. An OD_{578} of 1 corresponds to 107 µg/ml protein and a cell number of 10^8 cells/ml (Miller, 1972).

3.5.4 Growth curves and viability spot test

To investigate growth behaviour of strains under certain conditions an overnight culture of the strain was washed three times and diluted in fresh medium. The optical density of the culture was determined and aliquots were taken for Western blot analysis and/or viability spot tests. Depending on the experiment different dilution factors, media and inducers were chosen. Details of the growth and dilution conditions are given in the respective chapters in the result part.

For the viability spot test a serial dilution from 10^{-2} to 10^{-8} was made from the taken aliquots and 10 µl of the dilutions were dropped on NZA agar plates containing an appropriate antibiotic. After incubation at 37°C the number of surviving cells per dilution step was determined.

3.5.5 Strain construction via bacteriophage P1

The bacteriophage P1 was used to transfer selectable DNA fragments from one *E. coli* strain to another. P1 transduction was performed according to the method described by Silhavy *et al.* (Silhavy *et al.*, 1984).

3.5.5.1 Growing of a P1-Lysate

The overnight culture of the donor strain was diluted 1/100 in 5 ml fresh medium and was grown to early logarithmic phase (OD_{578} 0.3-0.4). Then 5 mM $CaCl_2$ and 10 µl or 100 µl of a $P1_{vir}$-lysate that was grown on strain MC4100 were added. The infected culture was grown with aeration at 37°C until lysis occurred. This was the case usually after 3 to 4 hours and visible as the culture became clear. A small amount of chloroform was added to kill remaining cells. After vortexing for a few seconds incubation at 37°C was continued for 10 minutes. The cell debris was pelleted by centrifugation for 5–10 minutes at room temperature (4000 x g) and the supernatant was collected in a screw-neck glass tube. The lysate was stored at 4°C.

3.5.5.2 Titration of a P1-Lysate

In order to quantify the phage titer of a lysate a serial dilution (10^{-2} to 10^{-8}) of that lysate was made in 10 mM $MgSO_4$. 100 µl of an overnight culture of MC4100 were mixed with 2.5 ml TB-topagar containing 5 mM $CaCl_2$ and poured on a LB agar plate. After the plate had dried 10 µl of each dilution were dropped on the plate and incubated over night at 37°C. The titer was determined as the number of phages per plated volume multiplied with the dilution coefficient.

3.5.5.3 P1-Transduction

The overnight culture of a recipient strain was diluted 1/50 in 5 ml fresh medium and grown to logarithmic phase (OD_{578} 0.5–0.7). Cells were pelleted by centrifugation and resuspended in 1.5 ml 100 mM $MgSO_4$ containing 5 mM $CaCl_2$ (final concentration). Different amounts of P1-lysate (1, 10, 100 µl) were added to aliquots of 0.5 ml cell-suspension. After incubation for 20 minutes at room temperature 0.2 ml of 1 M sodiumcitrate were added to prevent further absorption of virulent phages and the cells were pelleted by centrifugation. The pellet was resuspended in 1 ml LB medium containing 20 mM sodiumcitrate and placed at 37°C for 1 hour for phenotypic expression. Cells were pelleted by centrifugation again and plated on selective medium containing 20 mM sodiumcitrate and incubated over night at 37°C.

3.5.6 Integration of gene-constructs into the *E. coli* chromosome via bacteriophage λInCh

The bacteriophage λInCh was used to transfer plasmid-encoded *secA*-constructs to the *E. coli* chromosome. The phage contains a kanamycin resistance allele between two regions of homology: a part of the *bla* (ampicillin resistance) gene and a "near ori" region. These regions are homologues to sequences on pUC-derived or pBR-derived plasmids respectively. The transfer of the constructs was performed according to the methods described by Dana Boyd (Boyd *et al.*, 2000).

3.5.6.1 Growth of a λInCh lysate

λInCh contains an amber mutation in the S gene, which is required for normal lysis, and a temperature sensitive mutation in the repressor cI. Lysogens can be induced in strains that suppress the S amber mutation as well as in nonsuppressor strains. An overnight culture grown at 30°C of the *supF* strain DHB6500 or the nonsupressor host strain MC4100Δ*ara* was diluted 1/50 in LB medium with 5 mM $MgSO_4$ and grown to early log phase at 30°C. The culture was incubated at 42°C for 15 minutes to induce lysogens and incubation was

continued at 37°C with aeration. In the supF strain lysis occurred after about 1 hour. 200 µl of chloroform were added and the cell debris was pelleted by centrifugation for 10 minutes at room temperature (4000 x g). The supernatant was collected in a screw-neck glass tube. The lysate was stored at 4°C.

After 3 to 5 hours of incubation the nonsuppressor cells were pelleted by centrifugation and concentrated 10-fold by resuspension in LB medium with 5 mM $MgSO_4$. 200 µl of chloroform were added and the culture was incubated for additional 10 minutes at 37°C to lyse the cells. Debris was pelleted and the supernatant was collected as described above.

3.5.6.2 Titration of λInCh-lysates

In order to determine the phage titer of a λInCh-lysate the supF strain SM551 was grown to early logarithmic phase in LB medium with 5 mM $MgSO_4$ and 0.2% maltose. Serial dilutions of the lysate from 10^{-5} to 10^{-8} were made in LB medium containing 5 mM $MgSO_4$. 0.1 ml of every dilution step was mixed with a 0.1 ml aliquot of the SM551 culture and incubated for 10 minutes at 37°C. 2.5 ml of warm TB-topagar were added to the mixture and poured on LB agar plates containing 5 mM $MgSO_4$.

Plates were incubated over night at 37°C. The phage titer was determined as follows:

Phages/ ml= number of plaques/ volume (100 µl) x dilution coefficient

3.5.6.3 Infection in liquid culture and recombination between plasmid and λInCh DNA

SM551 or the nonsuppressor strain DHB4 was transformed with the plasmids containing the *secA*-constructs. An overnight culture of this strain was diluted 1/50 in fresh LB medium with 1 mM $MgSO_4$ and grown to mid logarithmic phase. Cells were pelleted by centrifugation and resuspended in 1/10 of the original volume in 10 mM $MgSO_4$. 10 µl of the λInCh lysate were added. After incubation for 15 minutes at room temperature the cells were diluted 10 fold in LB with 5 mM $MgSO_4$ and were grown at 37°C for 1 hour.

Cells were plated on LB agar plates containing ampicillin and kanamycin at 30°C or below. Colonies were purified on plates with ampicillin and tested for resistance to both antibiotics. In the next step a lysate of a purified and tested colony was grown as described in 3.5.6.1. The resulting lysates were termed λInCh-X, with X standing for the SecA amino acid that is followed by a TEV protease cleavage site.

3.5.6.4 Selection of ampicillin resistant lysogens

The lysate obtained above (3.5.6.3) contained a minority population of phages with a resistance to ampicillin and a sensitivity to kanamycin. These prophages contained the complete *bla* gene and the respective plasmid-derived *secA*-construct as a result of two recombination events at the homology sites. To select for these ampicillin resistant lysogens the strain MC4100Δ*ara* was infected with a λInCh-X lysate. 100 µl of an overnight culture of MC4100Δ*ara* was resuspended in 10 mM $MgSO_4$ and infected with 10 µl of undiluted, 1/10 and 1/100 diluted lysate. After incubation for 20 minutes at room temperature cells were plated on LB agar plates with 25 µg/ml ampicillin and incubated overnight at 30°C. Single colonies were purified and tested for ampicillin resistance and sensitivity to kanamycin.

3.5.6.5 Selection of temperature independent recombinants

The strain obtained above is temperature sensitive because at 42°C the induction of the prophage kills the host. However, λInCh was constructed with a 800 bp region that is homologues to a region on the bacterial chromosome near the attachment site. As a result of a recombination event between these "near-att" regions inside and outside the prophage most of the phage DNA is lost and the resulting recombinants are no longer temperature sensitive.

Ampicillin resistant, kanamycin sensitive colonies from the above strain were streaked out on agar plates and growth at 42°C was tested. Colonies that grew at high temperature were purified again at 30°C to purify away from S+ revertant phages and were tested again for ampicillin resistance and temperature independence.

3.6 Molecular biological methods

3.6.1 Transformation

3.6.1.1 TSS Transformation

Plasmid DNA was introduced in bacterial cells via TSS (*t*ransformation and *s*torage *s*olution) transformation (Chung *et al.*, 1989). A cell aliquot was taken early in the logarithmic phase (OD_{578} 0.3-0.4), mixed with an equal volume of ice-cold 2x TSS (usually 50 µl, composition see below) and stored on ice. The cells are immediately competent. Plasmid DNA (0.5-1 µg) was added and cells were further incubated for 20 minutes on ice. Phenotypic expression occurred for up to 1 hour at 28°C or 37°C after addition of 1 ml medium to the transformation reaction. The cells were grown overnight on selective plates containing an appropriate antibiotic.

2xTSS

PEG-6000	20% (w/v)
DMSO	10% (w/v)
$MgSO_4$	100 mM

PEG-6000 was dissolved in dH_2O and autoclaved prior to the addition of DMSO and $MgSO_4$. The solution was stored at 4°C.

3.6.1.2 Electroporation

To introduce ligated DNA plasmids and linearised DNA fragments into bacterial cells a high-efficiency transformation method relying on pulsed electrical fields was used (Wong and Neumann, 1982).

For the preparation of electro-competent cells an overnight culture was diluted 1/100 in 1 litre LB medium. Cells were grown at 37°C or 28°C to an OD_{578} of 0.5-1.0 and chilled on ice for 30 minutes. Cells were harvested by centrifugation in a cold GSA rotor at 4000

rpm for 5 minutes at 4°C. The supernatant was discarded and pellets resuspended in 1 volume ice-cold millipore H_2O (double distilled and filtered through a millipore filter apparatus). In a second and a third step cells were harvested by centrifugation as described above and the pellet was first resuspended in 1/2 volume ice-cold millipore H_2O and then in 1/50 volume ice cold and sterile 10% glycerol. The cells were then pelleted by centrifugation in a cold SS34 rotor at 5000 rpm for 5 minutes at 4°C and resuspended finally in 1/500 volume of 10% glycerol. Aliquots of 50 µl were quickly frozen in liquid nitrogen and stored at – 80°C.
For electroporation cells were thawed on ice and 1 µl of DNA was added to an aliquot of 50 µl cells before transferring the cells into a cold 2 mm electroporation cuvette. The Micro Pulser (Bio-Rad) was used according to the Bio-Rad Electroporation Application Guide (Cat.No.165-2100) with an electric pulse of 2.5 kV. 1 ml LB or NZA medium was added to the cells immediately. After incubation at 37°C for 1 hour the cells were plated on agar plates containing selective antibiotics.

3.6.2 Plasmid DNA preparation

Plasmid DNA was isolated using kits from QIAGEN. For low yield (up to 15 µg) of DNA 3-5 ml of an overnight culture were prepared following the protocol of the QIAprep® Miniprep handbook. For higher yields of DNA (up to 100 µg) 25 ml overnight culture were prepared according to the QIAprep®Midi protocol.

3.6.3 Enzymatic modification of plasmid DNA

3.6.3.1 Restriction digestion

Plasmid DNA was digested with restriction endonucleases for cloning and for proving proper fragment sizes. According to the description of the supplier each digestion reaction was performed in an appropriate buffer and at optimal temperature for each enzyme. Digestion reactions were composed as follows:

0.5 –1 µg DNA for restriction mapping or 5-10 µg DNA for cloning respectively
1/10 restriction buffer (provided by supplier)
5-10 units enzyme
100 µg/ml BSA if required
Sterile millipore H_2O was added to adjust the volume to 20 µl or 50-100 µl for cloning.

The digestion reaction was heat inactivated for 20 minutes at 65°C or 80°C depending on the restriction enzyme.

3.6.3.2 Generating blunt ends

Filling recessed 3'-termini

Recessed 3'- termini (5' overhangs) of digested DNA fragments were filled using *E. coli* DNA polymerase I large fragment (Klenow). To generate blunt ends 1 µl of dNTP solution (1 mM each dNTP in dH_2O) and 1 µl (5 units/µl) of Klenow fragment were added to a 20 µl digestion reaction and incubated for 20 to 30 minutes at room temperature. No specific buffer was used as Klenow fragment is active in all NEB buffers. The Klenow fragment was heat inactivated for 20 minutes at 75°C.

Removal of 3' overhangs

T4 DNA polymerase has a 3'-5' exonuclease activity and was used to generate blunt ends by removal of 3' overhangs. A 20 µl digestion reaction was incubated with 2 µl dNTPs (10 mM) and 2 µl T4 DNA polymerase. The reaction was placed into a water bath and temperature was cooled down from 18°C to 12°C within 15 minutes. The DNA polymerase was heat inactivated for 15 minutes at 75 °C

3.6.3.3 Dephosphorylation

In cloning processes the religation of vector fragments with identical termini was prevented by dephosphorylation. Alkaline phosphatase removes the 5' phosphates from both termini and therefore minimises recircularisation of the linear DNA fragment. Either

calf intestine alkaline phosphatase (CIAP) or shrimp alkaline phosphatase (SAP) were used to catalyse this reaction.

Dephosphorylation by CIAP

After restriction digestion 1 µl of calf intestine alkaline phosphatase (1 unit/µl stock) and 1/10 volume dephosphorylation buffer were added to the digested DNA. For sticky end ligations the dephosphorylation reaction was incubated for 30 minutes at 37°C. For blunt end ligations the reaction was incubated for additional 30 minutes at 56°C. The reaction was inactivated by adding 1/10 volume of 0.5 M EDTA (pH 8.0) and incubation for 20 minutes at 70°C. To achieve complete inactivation the DNA was loaded on an agarose gel and extracted from it.

Dephosphorylation by SAP

1 µl of shrimp alkaline phosphatase (1 unit/µl stock) and 1/10 volume dephosphorylation buffer were added to the DNA restriction reaction. This mixture was incubated for 30 minutes at 37°C to dephosphorylate sticky ends and 60 minutes at 37°C for the dephosphorylation of blunt ends. SAP was heat inactivated for 15 minutes at 65°C.

3.6.3.4 Ligation of DNA fragments

After extraction of the DNA fragments from an agarose gel they were ligated via T4 DNA ligase. This enzyme catalyses the joining of two DNA strands between the 5'-phosphate and the 3'-hydroxyl group. Ligation occurred either between cohesive ends or blunt ends. For the ligation reaction the vector and insert fragments were usually used in a ratio of 1/1, 1/3 or 1/5. 10x ligase buffer, ligase (0.1-1 unit) and sterile millipore H_2O were added to a final volume of 15 µl. Ligation reactions with sticky ends were incubated overnight at 4°C in a water bath placed in a polystyrene box. The start temperature in the water bath was 21°C. During incubation time the temperature slowly declined. Blunt end ligations were incubated overnight at 15-20°C.

3.6.4 Separation of DNA fragments on agarose gels

After restriction digestion DNA fragments were separated on 0.7 to 1.0 % (v/w) agarose gels in TAE buffer at a constant voltage of 80 V. Agarose concentration depended on the length of the DNA fragments. 1/8 volume sample buffer was added to the DNA prior to loading on the gel. The DNA-agarose gel was dyed in an ethidium bromide bath containing 1 µg ethidium bromide per ml TAE buffer. Ethidium bromide interchelates with DNA and is visible in UV light. The fragment length was compared to a basepair standard marker (λ-DNA *BstEII* digest).

Sample buffer

Bromphenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in dH_2O	30%

TAE buffer

Tris Base	40 mM
EDTA (pH8.0)	1 mM
Acetic acid	0.12% (v/w)

3.6.5 Extraction of DNA fragments from agarose gels

DNA fragments were excised from agarose gels with a scalpel on a UV light table. The extraction of the DNA from the agarose slice was performed using QIAquick® Gel Extraction kit according to the provided protocol.

3.6.6 Polymerase chain reaction (PCR)

PCR was used for cloning and to identify proper inserted *secA*-constructs in the *E. coli* chromosome.

3.6.6.1 PCR protocols

Pfu DNA polymerase was used for cloning, i.e. the amplification of plasmid-derived DNA fragments. The primers were designed with the required restriction sites. *Taq* DNA polymerase was used to prove and identify inserted DNA fragments in plasmids or to prove the proper insertion of *secA*-constructs in the chromosome. In the latter case a colony-PCR was performed using whole cells. A PCR sample was composed as follows:

PCR with *Pfu* DNA polymerase

Millipore H_2O	40 µl
10x *Pfu* buffer (supplied)	5 µl
DNA template (0.1 µg/µl)	1 µl
Primer 1 (0.5 µg/µl)	1 µl
Primer 2 (0.5 µg/µl)	1 µl
dNTPs (25 mM each)	1 µl
Pfu DNA polymerase	1 µl

PCR with *Taq* DNA polymerase

Millipore H_2O	77 µl or 59 µl*
10x buffer (supplied)	10 µl
$MgCl_2$ (25 mM)	6 µl
100x BSA (10 mg/ml)	1 µl
DNA template (0.1 µg/µl)	1 µl or cell suspension 20 µl*
Primer 1 (0.5 µg/µl)	1 µl
Primer 2 (0.5 µg/µl)	1 µl
dNTPs (10 mM each)	1 µl
Taq DNA polymerase	1 µl

*For colony-PCR one bacterial colony was picked from an agar plate and resuspended in 100 µl millipore H_2O. From this suspension 20 µl were used in a PCR reaction.

After denaturing at 94°C for 1-5 minutes 30 reaction cycles were performed. The annealing temperature was chosen 4-5°C below the melting temperature of the primers and primer were extended at 72°C. The extension time depended on the length of the fragment and the DNA polymerase (*Pfu*: 2 minutes/1 kb and *Taq*: 1 minute/1 kb).

PCR cycle

94°C	1 min
55-65°C	1 min
72°C	x min

The cycles were followed by an additional extension time of 7 minutes at 72°C.

3.6.6.2 Purification of PCR products

PCR products were purified using QIAquick® PCR purification kit according to the provided protocol.

3.6.6.3 Oligonucleotide-primers

Primers for different experiments are listed in the tables below.

Name	**Sequence**
3pCC6*MluI*	5'- CGA CGC GTT CAC CGT CAT CAC CGA AAC G -3'
3pZS*AclI*	5'- CCC AAC GTT TTC TAT CGC CTT CTT GAC GAG T -3'
3spec*AclI*	5'- CCC AAC GTT ACG AAT TCG AGC TCG GT -3'
5pCC6*KpnI*	5'- CGG GGT ACC ATG CAA GTT TCA GTT GAA ACC ACT CAA G -3'
5pZS*AclI*	5'- CCC AAC GTT CAA TCC ATC TTG TTC AAT CAT -3'
5spec*AclI*	5'- CCC AAC GTT GCA TGC CTG CAG GTC -3'

Tab. 3.8 Oligonucleotide-primers for the cloning of plasmids pTH9 and pTH10.

Name	Sequence
3Kan	5'- GCA ACA TCT TGC ATT ACT GGA TAC G -3'
3PCS	5'- CGC TGA CTG GAA GTA CAG GTT TTC -3'
3*secA*end	5'- TCT GCG GCC GCT TAT TGC AGG CGG CCA TGG CAC T -3'
3*secA*229	5'- CGT CTA GAT GCC GGG CCG GAA ATG ATC AGC -3'
3*secA*296	5'- CGT CTA GAC TCC CCT TCA TCC ATG ATG CC -3'
3*secA*452	5'- CCG GCT GGC CTT TCG CAG TAC G-3'
3*secA*518	5'- CGT CTA GAG CTA CCA CCG AGC GCA ATA TCT GTA -3'
5gene*X*	5'- GCA TTA CGC TGA CTT GAC GGG ACG G-3'
5*secA*start	5'- GGG GTA CCG CTA ATC AAA TTG TTA ACT AAA GTT TTC GG -3'
5*secA*230	5'- AGA GCG GCC GCT GAA GAC AGC TCG GAA ATG T -3'
5*secA*297	5'- AGA GCG GCC GCG TCT CTG TAC TCT CCG GCC ACC ATC -3'
5*secA*519	5'- AGA GCG GCC GCC TGG CAG GCA GAA GTT GC -3'
5up*secA*	5'- GAA ACA GCT ATG ACC ATG ATT ACG -3'

Tab. 3.9 Oligonucleotide-primers for the verification of *secA*-constructs and cloning of PCS into *secA*

Name	Sequence
3*secA*229*SmaI*	5'- TCT CCC GGG TGC CGG GCC GGA AAT G -3'
3*secA*295*SmaI*	5'- TCT CCC GGG CCC TTC ATC CAT GAT GCC C -3'
3*secA*296*SmaI*	5'- TCC CCC GGG CTC CCC TTC ATC CAT GAT -3'
3*secA*518*SmaI*	5'- TCT CCC GGG GCT ACC ACC GAG CAC AAT ATC -3'
5tet*SmaI*	5'- TCC CCC GGG GAA AAC CTG TAC TTC CAG TCA GCG -3'

Tab. 3.10 Oligonucleotide-primers for amplifying plasmids pSecA229-518 without the 12 amino acid sequence.

Name	Sequence
3*BglII*42	5'- GAA GAT CTT CAG CCG ATC TTG ATG AGA CC -3'
3*HindIII*42	5'- CCC AAG CTT GCA TGC GTG CGT CAG CCG ATC T -3'
3*malF*-L1*XbaI*	5'- GCT CTA GAC TGA ATG CCT TCG TCG GTA AAG ACG -3'
3*malF*-S1*XbaI*	5'- GCT CTA GAG CGC CAG GCG TAG GCT TTA CGA TTG -3'
3*phoAXbaI*	5'- GCT CTA GAT TTC AGC CCC AGA GCG GCT TTC -3'
5*malF*-L1*NcoI*	5'- CGG ACC ATG GGA GAT GTC ATT AAA AAG AAA CAT TGG TGG C -3'
5*malFNheI*	5'- CTA GCT AGC CGT CCT GGA ATG AGG AAG AAC C -3'
5*NcoI*42-gly	5'- CGG ACC ATG GGA TCG CAG GCT GCT CTG AAC -3'
5*NheI*42	5'- CTA GCT AGC GAA TTC TGG AAC TGG CGG ACT AAT -3'
5*phoANheI*	5'- CTA GCT AGC TCA GCG ATA CAT GGA GAA AAT AAA GTG AAA C -3'
5*phoASmaI*	5'- TCC CCC GGG AAA CAA AGC ACT ATT GCA CTG GCA C -3'

Tab. 3.11 Oligonucleotide-primers for cloning of plasmids pTH20, pTH23, pTH24, pTH25, pTH28 and pTH29

Name	Sequence
3KD46*PstI*	5'- AAC TGC AGT GAG CGC AAC GCA AT -3'
5KD46*PstI*	5'- AAC TGC AGG TCG ACT CTA GAG GA -3'

Tab. 3.12 Oligonucleotide-primers for cloning of plasmid pKD46spec

Name	Sequence
fixexpressleft	5'- GCT TAA TCA GTG AGG CAC CTA TC -3'
fixexpressright	5'- CGC GGA TCC CAT GGG GAG ATG GCT TAA ATC CTC CAC C -3'

Tab. 3.13 Oligonucleotide-primers for repairing plasmid pMIC16

3.6.7 Cycle sequencing

The affected segments of plasmids pTH12, pTH13, pTH14 and pTH15 carrying the shortened *secA* constructs derived from plasmids pSecA229, pSecA295, pSecA296 and pSecA518 were sequenced using the Big Dye terminator cycle sequencing V2.0 kit (ABI). The sequencing reaction was composed as follows:

Big Dye terminator V2.0 ready reaction mix	1 µl
Better buffer (Microzone)	5 µl
Double stranded DNA	250-300 ng
Primer	2.4 pmol

Sterile millipore water was added to a final volume of 15 µl.

The primers used for the sequencing of the respective plasmids are listed in Table 3.14.

Plasmid	**Name**	**Sequence**
pTH12	5seq229	5'- ATCCTGATCGATGAAGCGCGTACACCG -3'
pTH13, 14	5seq295/6	5'- CTGGTGCTGATTGAAGAACTGCTGGTG -3'
	3seq295/6	5'- CGTGGTGCATCAGCATGATGTTGG -3'
pTH15	5seq518	5'- GCGACCAATATGGCGGGTCGT -3'

Tab. 3.14 Oligonucleotide primers used for cycle sequencing for the listed plasmids. Primers 5seq are for reading in 5' to 3' direction, primer 3seq for reading in 3' to 5' direction.

25 cycles were performed in a PCR machine as follows:
rapid ramp (1° /sec) to 96°C for 10 seconds
rapid ramp (1° /sec) to 50°C for 5 seconds
rapid ramp (1° /sec) to 60°C for 4 minutes.

To remove unincorporated dye-labelled terminators the samples were precipitated in 60% isopropanol at room temperature for 10 minutes. The precipitate was centrifuged for 30 minutes at 4°C in an Eppendorf centrifuge. The pellets were rinsed with 75% isopropanol. After a second centrifugation step as before the pellets were dried over night at 37°C in darkness. The following sequence reading was performed by Gareth Lewis (Cardiff University).

3.6.8 Inactivation of chromosomal *secA* using PCR products and λ Red recombinase

The chromosomal gene *secA* was eliminated by using PCR products according to the method described by Datsenko and Wanner (Datsenko and Wanner, 2000). These PCR products contained a selectable antibiotic resistance cassette (kanamycin), which was flanked by FRT sites (FLP recognition target). Both ends of this DNA fragment were synthesised with short sequences that were homologues to regions adjacent to *secA*. The λ Red recombinase system was used to perform the recombination between the PCR product and the chromosomal sequence resulting in the loss of the targeted gene. The remaining kanamycin resistance gene was eliminated via recombination between the flanking FRT sites mediated by FLP recombinase. The helper plasmids expressing the Red recombinase system and FLP recombinase respectively were cured by growth at 42°C and 37°C as they contained temperature sensitive replicons.

3.6.8.1 Generating the PCR product for secA elimination

Pfu polymerase was used for the amplification of the required DNA fragment. Plasmid pKD4 containing the FRT flanked kanamycin cassette was used as a template. Primers 5KD4 and 3KD4 were designed with a 35-nt or 37-nt extension respectively (H1 and H2) that are homologues to sequences adjacent to *secA*. H1 and H2 were followed by 21-nt or 18-nt priming sites for pKD4 called P1 and P2 respectively (Table 3.15). The PCR reaction was performed as described in chapter 3.6.6.1 with 1 cycle at 94°C for 2 minutes and 30 cycles with 94°C for 40 seconds, 56°C for 1 minute and an extension time of 3 minutes at 72°C. 5 µl of DMSO were added to a final volume of 50 µl PCR reaction. The 1.4 kb PCR product was purified using QIAquick® purification kit and then *Dpn I* digested for 2 hours to eliminate traces of the template plasmid. The restriction reaction was gel-purified on an agarose gel and the PCR product was resuspended in elution buffer (10 mM Tris pH 8.0).

Name	Sequence	
	H (near *secA* homology)	**P (pKD4 priming)**
3KD4	5'- CAG AAT CCT GCG CCT TTT ACT TCA ACA GTT AGC TTT TTA TGA ATA TCC TCC TTA G -3'	
5KD4	5'- GGC ACG CCG TCT GAA AAG GGT TAT CGC ATT GAT TAG TGT AGG CTG GAG CTG CTT CG -3'	

Tab. 3.15 Oligonucleotide-primers for amplifying FRT flanked kanamycin resistance from plasmid pKD4 with near *secA* homology sequences.

3.6.8.2 Elimination of chromosomal secA

Strains carrying a chromosomal *secA*-construct in the attachment site were transformed with the temperature sensitive Red recombinase helper plasmid pKD46spec. Transformants were grown over night in NZA medium at 28°C. Cultures were diluted 1/100 in SOB medium containing spectinomycin and 0.2% L-arabinose to induce the Red recombines system and grown at 28°C to an OD_{578} of 0.6 to 0.8. Cells were made electro-competent and concentrated 500-fold by washing three times in ice-cold millipore H_2O as described in chapter 3.6.1.2. Cells were resuspended in 10% ice-cold glycerol. 3 µl of purified PCR product was electroporated into 100 µl competent cells. 1 ml of SOC medium was added immediately and the mixture was incubated for 1 h at 37°C. One-half of the cells was plated at 37°C on agar plates containing 100 mM kanamycin. The other half was saved at room temperature over night and spread on kanamycin agar plates if no colony grew from the first spread. Kanamycin resistant transformants were purified once nonselectively at 37°C and then tested for spectinomycin sensitivity to confirm the loss of the helper plasmid. A few colonies still showing growth on spectinomycin were purified at 42°C and tested again.

3.6.8.3 Test for Red recombinase induction

To test the induction of the Red recombinase system an overnight culture of a strain transformed with plasmid pKD46spec was diluted 1/100 in NZA medium containing either 0.2% arabinose or no inducer and cells were grown for 4 hours at 28°C. Protein samples were taken and loaded on a 12% SDS-polyacrylamide gel as described in chapter

3.7.1. Induced and noninduced samples were compared by staining the protein bands with Coomassie Blue as described in chapter 3.7.2.

3.6.8.4 PCR Verification of strains

The correct insertion of the FRT-flanked kanamycin resistant gene was tested by performing colony-PCR using *taq* DNA-polymerase as described in chapter 3.6.6.1. Primers 3KD4 and 5KD4 (Table 3.15) were used to confirm the presence of the kanamycin cassette. Primers 5gene*X* and 3into*secA* (Table 3.16), priming sequences adjacent to *secA*, were used to confirm the correct position of the kanamycin cassette and the loss of *secA* respectively.

Name	**Sequence**
3into*secA*	5'- CTT CCT GAA GTT CAC GCA CCA C -3'
5gene*X*	5'-GCA TTA CGC TGA CTT GAC GGG ACG G-3'

Tab. 3.16 Oligonucleotide-primers for amplifying FRT flanked kanamycin resistance with *secA* homology sequence

3.6.8.5 Elimination of the FRT flanked kanamycin-cassette and PCR confirmation

Strains containing the FRT flanked kanamycin cassette instead of chromosomal wt *secA* were transformed by electroporation with plasmid pCP20 that shows temperature sensitive replication and thermal induction of FLP recombinase. Transformants were selected on NZA chloramphenicol agar plates at 28°C. Colonies were purified once nonselectively at 42°C and then tested for the loss of pCP20 and the Kan-cassette by selecting for chloramphenicol and kanamycin sensitive colonies.

The loss of the kanamycin cassette was verified by colony-PCR using *taq* DNA-polymerase and primers 3into*secA* and 5gene*X*.

3.6.9 DNA-macroarrays

Changes of global gene-expression patterns as a response to Ffh-, FtsY- or SecA-depletion respectively were studied on a transcriptional level by performing Panorama™ *E. coli* gene macroarrays (Sigma Genosys).

3.6.9.1 Stabilisation and Isolation of total RNA

Enzymatic degradation of RNA and gene induction during handling of the samples can lead to artefacts in the gene expression profile. To minimise this problem RNA was stabilised using RNAprotect Bacteria Reagent (QIAGEN) prior to RNA isolation. Two volumes of the reagent were added to one volume of bacterial culture immediately after removing the culture from the incubator. Total RNA was isolated using RNeasy®Mini Kit (QIAGEN). For an average yield of 13 µg RNA which was sufficient for further steps 1.6 – 2 ml of mid-logarithmic phase culture (OD_{600} 0.25-0.4) were prepared following the instructions of the RNeasy®Mini Handbook. According to the protocol an average yield of 55 µg RNA can be isolated from the maximum amount of starting material of 1 x 10^9 bacterial cells per column. Residual amounts of DNA were removed by using the RNase-free DNase set from QIAGEN during RNA isolation according to the suppliers instructions.

3.6.9.2 Quantitation, integrity and storage of total RNA

The concentration of total RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml in water. Therefore, the RNA concentration of the sample was calculated as follows:

40 x A_{260} x dilution factor = µg RNA/ml

The integrity and the amount of genomic DNA concentration was tested by non-denaturing agarosegel electrophoresis and ethidium bromide staining. 2-3 µg RNA were mixed with sample buffer (see chapter 3.6.4) and loaded on a 1.2% agarose gel

containing 1 µg/ml ethidium bromide. To avoid RNase contamination the gel chamber and other plastic-ware was cleaned with a detergent solution, rinsed with water and dried with 70% ethanol. The chamber was rinsed with sterile water and filled with sterile TAE buffer (see chapter 3.6.4). RNA samples which showed a negligible DNA-contamination (visible as a high molecular weight band) and clear bands of the 23S and 16S ribosomal RNA with an approximate ratio of 2/1 were stored in RNase free water at – 80°C.

3.6.9.3 Synthesis of labelled cDNA

Labelled cDNA was synthesised in a reverse transcriptase reaction by using *E. coli* cDNA labelling primers (Sigma Genosys) following the instructions of the Panorama™ *E. coli* gene array handbook.

In a first step the primers were annealed to the RNA template as follows:

Primer annealing reaction

2 µg total RNA	x µl
E. coli labelling primers	4 µl

Sterile RNase free distilled water was added to adjust the volume to 15 µl.

The reaction was placed in a thermal cycler and heated to 90°C for 2 minutes and then cooled down to 42°C over a period of 20 minutes. Once the mixture had reached 42°C the components for the cDNA labelling step were added:

Reverse transcriptase reaction

Annealing reaction	15 µl
1 x reverse transcriptase buffer	6 µl
333 µM dATP	1 µl
333 µM dGTP	1 µl
333 µM dTTP	1 µl
20 µCi [α-^{33}P] dCTP (2000 Ci/mmol)	2 µl
50 U AMV reverse transcriptase	2 µl (AMV = avian myeloblastosis virus)

Sterile RNase free distilled water was added to adjust the volume to 30 µl.

The cDNA labelling reaction was incubated for 3 hours at 42°C in a thermal cycler. The unincorporated nucleotides were removed from labelled cDNA by using MicroBio-Spin®6 Chromatography columns (Bio-Rad) following the manufacturer's protocol.

3.6.9.4 Hybridisation

The purified radioactively labelled cDNA was hybridised to the *E. coli* Panorama gene array (Sigma Genosys) which consists of a 12- by 24- cm positively charged nylon membrane on which DNA from all 4290 protein-coding ORFs (Blattner *et al.*, 1997) have been printed in dublicate at an equal mass (10 ng) per spot. The hybridisation was carried out in roller bottles in a hybridisation oven following the manufacturer's instructions. The array membranes were pre-hybridised for at least 1 hour at 65°C in 5 ml pre-warmed hybridisation solution. The entire labelled cDNA probe was added to 3 ml hybridisation solution and denatured at 95°C for 10 minutes. Then the array was hybridised with this solution for 16 hours at 65°C.

The arrays were washed three times for 3 minutes at room temperature with 50 ml wash solution and three times for 20 minutes at 65°C with 100 ml pre-warmed wash solution. The array membranes were then wrapped in plastic food wrap (Saran) and exposed to a PhosphorImager screen (Imaging Screen K, Bio-Rad) for 1-3 days.

For each experiment the same array membrane was rehybridised after stripping, either with the control or test probe. The arrays were stripped at 100°C for 30 minutes in stripping solution, wrapped in food wrap and exposed to a PhosphorImager screen over night.

<u>Solutions used for hybridisation and stripping</u>

SSPE 1x	NaCl	0.18 M
	NaH_2PO_4	10 mM, pH 7.7
	EDTA	1 mM

Denhardt's Reagent	Ficoll	0.02%
	Polyvinylpyrrolidone	0.02%
	BSA	0.02%
Hybridisation solution	SSPE	5x
	SDS	2%
	Denhardt' Reagent	1x
	Sonicated, denatured salmon testes DNA	100 µg/ml
Wash solution	SSPE	0.5x
	SDS	0.2%
Stripping solution	Tris, pH7.5	10 mM
	EDTA	1 mM
	SDS	1%

3.6.9.5 Analysis of array data

The exposed PhosphorImager screens were scanned at a 50-µm pixel resolution in a Molecular Imager®FX (Bio-Rad). Hybridisation intensity was quantified from the image file using Quantity One (Bio-Rad). A grid of volume circles corresponding to the spots on the array was overlaid on the image and for each spot the mean intensity of the pixels inside the volume boundary was calculated. Background was subtracted locally for each spot. The mean intensity values were then exported into Excel (Microsoft®) for further analysis and comparison. In a first step the average pixel intensity of a pair of duplicate spots was calculated. Then relative signals from the control and test array were normalised by representing the averaged spot intensity as a percentage of the total signal from all spots of the respective array. The ratio between the normalised averaged values from the test set (depleted cells) and the control set (undepleted cells) represented the fold change in gene expression. Ratios were $\log_2$-transformed and analysed using SAM

(Significance Analysis of Microarrays) to identify genes that were significantly up- or down-regulated. This statistical method was proposed by Tusher, Tibshirani and Chu (Tusher *et al.*, 2001) and the software was downloaded from the Stanford University web site http://www-stat.stanford.edu/~tibs/SAM.

SAM is based on gene-specific t-tests and permutation analysis. Each permutation involves sampling at random a sub population of the data set and identifying the highest t-test score. Here, 3000 permutations were performed for each experiment.

The program is able to analyse different types of experiments. In this case a one-class experiment was performed where the average test/control expression ratios were compared against a theoretical ratio of 1 (or rather the $\log_2$ transformed ratios were compared against $\log_2 1 = 0$). In a one-class response the t-test score, also called relative difference d_i for a gene i, is defined as follows: $d_i = r_i / (s_i + s_0)$; $i = 1,2,...p$ genes.

The numerator r_i is the average gene expression ratio of the gene i ($\log_2$ transformed).

The gene specific scatter s_i is the standard deviation of repeated measurements:

$s_i = \{\sum_j (x_{ij} - x_i)^2 / (n\,(n-1))\}^{1/2}$; $j = 1,2,...q$ samples and n = number of repeats. The small positive constant s_0 ensures that the variance of d_i is independent of gene expression. Details of s_0 are described in the user handbook of SAM (Chu *et al.*, 2002), which can be downloaded from the Stanford University web site mentioned above.

To identify potentially significant genes the program ranked the genes by magnitude of their d_i values. For each permutation a relative difference d_{iP} was also calculated and ranked. The expected relative difference d_{iE} was then defined as the average over the number of permutations. In a scatter plot the observed relative difference d_i was compared against the expected relative difference d_{iE}. Genes that were represented by points outside a freely adjustable threshold Δ, which is the difference from the $d_i = d_{iE}$ line, were called potentially significant. For each threshold the program computed a false discovery rate (FDR) to estimate the percentage of genes that were identified as significant by chance.

3.7 Biochemical methods

3.7.1 SDS – polyacrylamide gel electrophoresis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7%, 10% or 12% acrylamide gels respectively (Laemmli, 1970). Samples were prepared from overnight cultures or cultures that were diluted in fresh medium and induced for several hours. To ensure an equal amount of proteins in the samples, the cultures were adjusted to the same OD_{578}. 0.2 –3 ml of culture were pelleted by centrifugation for 5 minutes and 1x sample buffer containing DTT (as a reducing agent) and the anionic detergent SDS was added. The sample was vortexed for 1 minute to destroy the DNA and heated to 95°C for 10 minutes to denature the protein. The denatured polypeptides bind SDS and become negatively charged. The amount of bound SDS is proportional to the molecular weight of the protein and the SDS-protein complex migrates through the polyacrylamide gel according to the size of the polypeptide. As the complex is negatively charged it migrates to the positive electrode in an electric field. The gels were run at 25 mAmp for 1 hour. Prestained SDS-PAGE standards from Bio-Rad (low range and broad range) were used to identify the molecular weight of a protein.

Solutions for SDS-PAGE

Buffer A	Tris	181.7 g	pH 8.8 w/HCl
	SDS	4.0 g	
	volume adjusted to 1 litre with dH_2O		
Buffer B	Tris	60.6 g	pH 6.8 w/HCl
	SDS	4.0 g	
	volume adjusted to 1 litre with dH_2O		

Sample buffer 4x	Tris	0.24 M	pH 6.8 w/HCl
	SDS	8%	
	Glycerin	40%	
	DTT	30 mM added before loading to the gel	
Running buffer	Tris	20.25 g	pH 8.3 w/HCl
	Glycine	72.0 g	
	SDS	0.1%	
	volume adjusted to 5 litres with dH_2O		

Composition of SDS-polyacrylamide gels

	7%	**10%**	**12%**	**Stacking gel**
Buffer A	2500	2500	2500	-
Buffer B	-	-	-	2000
Acrylamide (30%)	2300	3300	4000	1200
H_2O	5200	4200	3500	4800
TEMED	10	10	10	20
APS (10% in H_2O)	25	25	25	40

Tab. 3.10 Composition of SDS- polyacrylamide gels. Volume in [µl].

3.7.2 Rapid Coomassie staining

Protein bands on polyacrylamide gels were stained with Coomassie Blue (Silhavy *et al.*, 1984). After SDS-gel-electrophoresis the gel was heated in solution A for 20–30 seconds in a microwave and incubated for 5 minutes on a shaker. Solution A was replaced by solution B containing less Coomassie Blue and treated in the same way. Solution B was discarded and solution C was added to the gel. After heating in the microwave the gel was incubated in solution C for 15 minutes.

To destain the background the gel was incubated in destaining solution for at least 1 hour at room temperature.

Solutions for Coomassie staining

Solution A	2-propanol	25%
	Acetic acid	10%
	Coomassie Blue	0.05%
Solution B	2-propanol	10%
	Acetic acid	10%
	Coomassie Blue	0.005%
Solution C	Acetic acid	10%
	Coomassie Blue	0.002%
Destaining solution	Acetic acid	10%

3.7.3 Western blot analysis

Specific proteins were identified immunologically by Western blotting (Towbin *et al.*, 1979). In this technique proteins that were separated on a SDS-PAGE were transferred electrophoretically from the gel to a PVDF membrane (polyvinylidene difluoride, Fluoro Trans, Pall) and probed with antibodies that react specifically with antigenic epitopes displayed by the target protein (Sambrook *et al.*, 1989).

The PVDF membrane was activated in 100% methanol for a few seconds. After the blotting of the proteins to the membrane in a Bio-Rad Mini Trans Blot® Cell apparatus for 60 minutes at 100 V the membrane was blocked for at least one hour in TBST buffer with 2% BSA to prevent unspecific cross reactions of the primary antibody. The membrane was incubated for 1-2 hours with the primary antibody diluted 1/10000 in blocking solution. The primary antibody is specific for the target protein and unbound antibody was washed out three times via incubation in ca. 10 ml TBST buffer for 5 minutes on a table shaker. The bound antibody was detected by a second antibody which was added 1/20000 diluted in TBST. The membrane was incubated for 30-60 minutes and then again

washed three times with 10 ml TBST. The membrane was washed once in 10 ml AP buffer for 10 minutes prior to staining. The membrane was stained in a solution containing XP. XP is a substrate of alkaline phosphatase, which is fused to the secondary antibody. Converted XP produces a blue precipitate on the membrane. The reaction was stopped by rinsing the membrane with H_2O.

Solutions for Western blotting

TBST buffer	Tris	50 mM	pH 7.5 w/HCl
	Tween-20	0.1%	
	NaCl	150 mM	
AP buffer	Tris	100 mM	pH 9.0 w/HCl
	NaCl	100 mM	
	$MgCl_2$	5 mM	
Transfer buffer	Tris	15 mM	
	Glycine	120 mM	
	SDS	10 mM	
	Methanol	20% (v/w)	
Staining solution	AP buffer	10 ml	
	NBT	66 µl (20 mg/ml in 70% DMF)	
	XP (BCIP)	66 µl (20 mg/ml in 100% DMF)	

3.7.4 Detection of biotinylated proteins by immunoblotting

Proteins that were fused to the biotinylatable domain from the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase (PSBT) were separated on a SDS-PAGE and transferred to a PVDF membrane by Western blotting. The membrane was blocked for at least 1 hour at room temperature in TBST buffer with 2% BSA and then incubated

for 1 hour with streptavidin-alkaline phosphatase diluted 1/3000 in TBST buffer. Streptavidin is a tetrameric protein which binds very tightly to the small biotin molecule. The membrane was washed 3 times in TBST buffer for at least 5 minutes and once in AP buffer prior to staining. Biotinylated proteins on the membrane were detected by exposing the membrane to the staining solution described in chapter 3.7.3 containing XP, the substrate for alkaline phosphatase. The reaction was stopped after about 30 seconds by rinsing the membrane in H_2O.

3.7.5 AP Assay

To determine the activity of alkaline phosphatase expressed under the control of the P_{malK} promoter on plasmid pTH2 an overnight culture of strain TH2 containing pTH2 was grown in LB medium with 100 µM ampicillin. This culture was diluted 1/20 in the same medium and various test media, that is LB medium containing ampicillin and 0.2% glucose or different concentrations of maltose respectively. The cells were grown at 37°C and samples were taken every hour. The actual OD_{600} was measured and adjusted to an OD_{600} of 0.3 before 0.9 ml of washing buffer (10 mM Tris/HCl pH 8, 150 mM NaCl) were added to these cells. Cells were washed twice in this buffer at room temperature and then 750 µl were used to determine OD_{600}.

0.9 ml of assay buffer (1 M Tris/HCl pH 8, 0.1 mM $ZnCl_2$) was added to 100 µl of washed cells and mixed gently. After the substrate of the enzyme, p-nitrophenyl Phosphate (0.4% pNPP in 1M Tris/HCl pH 8), was added the assay was mixed again and incubated at room temperature until a yellow colour had developed. The reaction was stopped by adding 120 µl K_2HPO_4 (2.5 M). Cells were pelleted by centrifugation for 5 minutes at room temperature with a table centrifuge. The AP activity was calculated by measuring the OD_{420} of the supernatant and inserting the value into the formula stated below:

$$\text{AP activity [units]} = \frac{OD_{420} \times 1000}{\text{time [min]} \times OD_{600} \times \text{cell volume [ml]}}$$

4 Results

4.1 A system to deplete and inactivate *E. coli* SecA *in vivo*

SecA is an essential component of the secretion machinery. A knock-out of the gene is lethal and not suitable for investigating SecA function. To obtain more information on additional potential roles of SecA it is important that SecA function can be switched off under distinct conditions so that the effects of the loss of SecA function can be investigated. Here, a system was established where *E. coli* cells were depleted of SecA by placing a chromosomal *secA*-construct under P_{lac} control. Additionally, SecA function was inactivated by transforming the protein into a substrate for a virus-derived protease (TEV protease) that was also expressed under a regulatable and tight promoter.

4.1.1 Replacing wt *secA* by chromosomal *secA*-PCS constructs under *lac* promoter control

In previous experiments several *secA*-constructs containing the heptapeptide cleavage site of TEV protease (PCS) were recombined on the chromosome of *E. coli* replacing wild-type *secA*. The PCS (E-N-L-Y-F-Q-S) was introduced into SecA behind the amino acids 195 and 252. The insertion did not interfere with normal SecA function. When TEV protease was expressed in these cells proteolysis of the constructs occurred and a secretion defect (measured as pre-OmpA accumulation) was shown as a result of proteolysis of SecA195 (Mondigler and Ehrmann, 1996; Mondigler, 1997). In these experiments the *secA*-constructs were still under wt *secA* promoter control. However, as described in chapter 2.2.3.3 translation of SecA is autoregulated. If the intracellular SecA level is low SecA dissociates from a binding site located on its own mRNA and allows further translation. To prevent a negative effect by *de novo* synthesis of SecA it is important to regulate the expression of the *secA*-constructs. Therefore, *secA*-PCS constructs were placed under the control of the *lac* promoter on pUC-derived plasmids

(Fig. 4.1). Plasmid pMKL18 carried the wt *secA* gene without the upstream *geneX* (*secM*) region. The plasmids pMM51, pMM52, pMM54 and pMM55 carried constructs of *secA* with introduced PCS behind the amino acids 195, 252 and 830 (Mondigler, 1997). These *secA*-constructs were inserted at the λ attachment site using the bacteriophage λInCh as a vehicle. The synthesis of wt SecA was then prevented by eliminating wt *secA* from the chromosome as described by Datsenko and Wanner (Datsenko and Wanner, 2000) using λ Red recombinase and an appropriate PCR product.

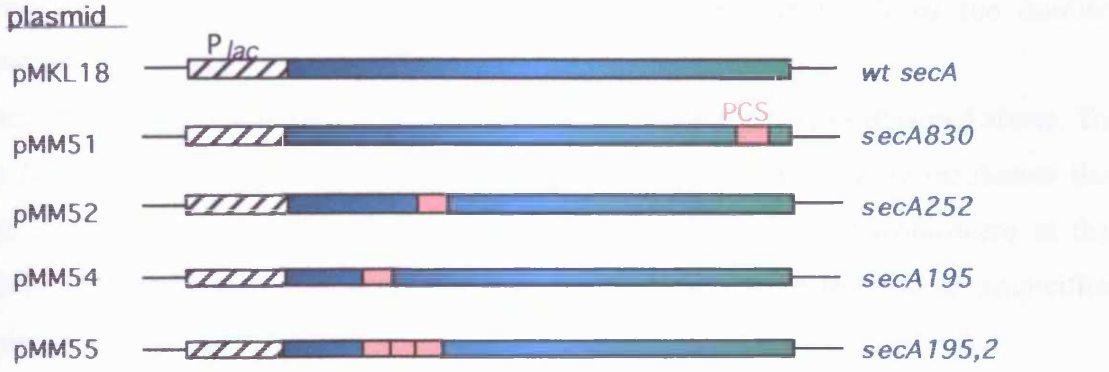


Fig. 4.1 Schematic view of wt *secA* and *secA*-constructs behind the *lac* promoter on plasmids pMKL18, pMM51, pMM52, pMM54 and pMM55 (Mondigler, 1997). TEV protease cleavage sites (PCS) are shown as red squares.

4.1.1.1 Transfer of secA-PCS constructs to the chromosome using λInCh as a shuttle system

The bacteriophage λInCh provides a shuttle system between plasmid and chromosome in *E. coli* to achieve low-level expression of a gene from a single copy (Boyd *et al.*, 2000). Using λInCh as a vector the *secA*-constructs were inserted at the λ attachment site in the *E. coli* chromosome via site directed recombination. There are two slightly different λInCh phages: λInChI possesses homologous sequences to pBR-derived plasmids and λInChII to pUC derived plasmids. These sequences are a region near the replicative origin, called "near-ori", and a fragment of the *bla* gene that encodes ampicillin resistance. As the pMM-plasmids carrying the *secA*-constructs as well as pMKL18 are

pUC-derived plasmids the λInChII phage was used. The transfer of the *secA*-constructs required three successive steps (Fig. 4.2).

First, during λInCh-infection of strain DHB4 which was transformed with the plasmids pMKL18 (wt *secA*), pMM51 (*secA*830), pMM52 (*secA*252), pMM54 (*secA*195) and pMM55 (*secA*195,2) respectively, a double recombination event was possible due to the two homologous regions. The lysates derived from this infection contained a minority of phages (about 1 in 1000) that were ampicillin resistant because the sequence of the *bla* gene had been completed. They were also kanamycin sensitive due to the exchange of the kanamycin resistance allele by the *secA*-construct as the result of the double recombination.

In a second step the strain MC4100Δ*ara* was infected with the lysates obtained above. To allow formation of lysogens the reaction was incubated at 28°C. During incubation the phage integrated via site-specific recombination into the *E. coli* chromosome at the λ attachment site between the genes *gal* and *bio*. Colonies were selected on ampicillin containing media at 28°C.

Integration of the phage is a reversible procedure. To anchor the *secA*-constructs stably at the λ attachment site "temperature-independent" colonies were selected in a third step. λInCh possesses a temperature sensitive allele of the lambda repressor cI. At high temperature (42°C) this repressor is inactivated and the genes responsible for the lytic cycle are induced. The replication of the phage then kills the host.

However, in 1/10,000 cases another recombination occurred between a 800 bp fragment of the chromosome near the attachment site and the identical fragment in the phage genome (called "near-att"). As a result of this recombination event most of the phage genome is deleted. Cells in which this recombination event had taken place were selected via growth at 42°C. Temperature-independent and ampicillin resistant colonies were selected for all four SecA-PCS constructs as well as for the plasmid-derived SecA under *lac* promoter control.

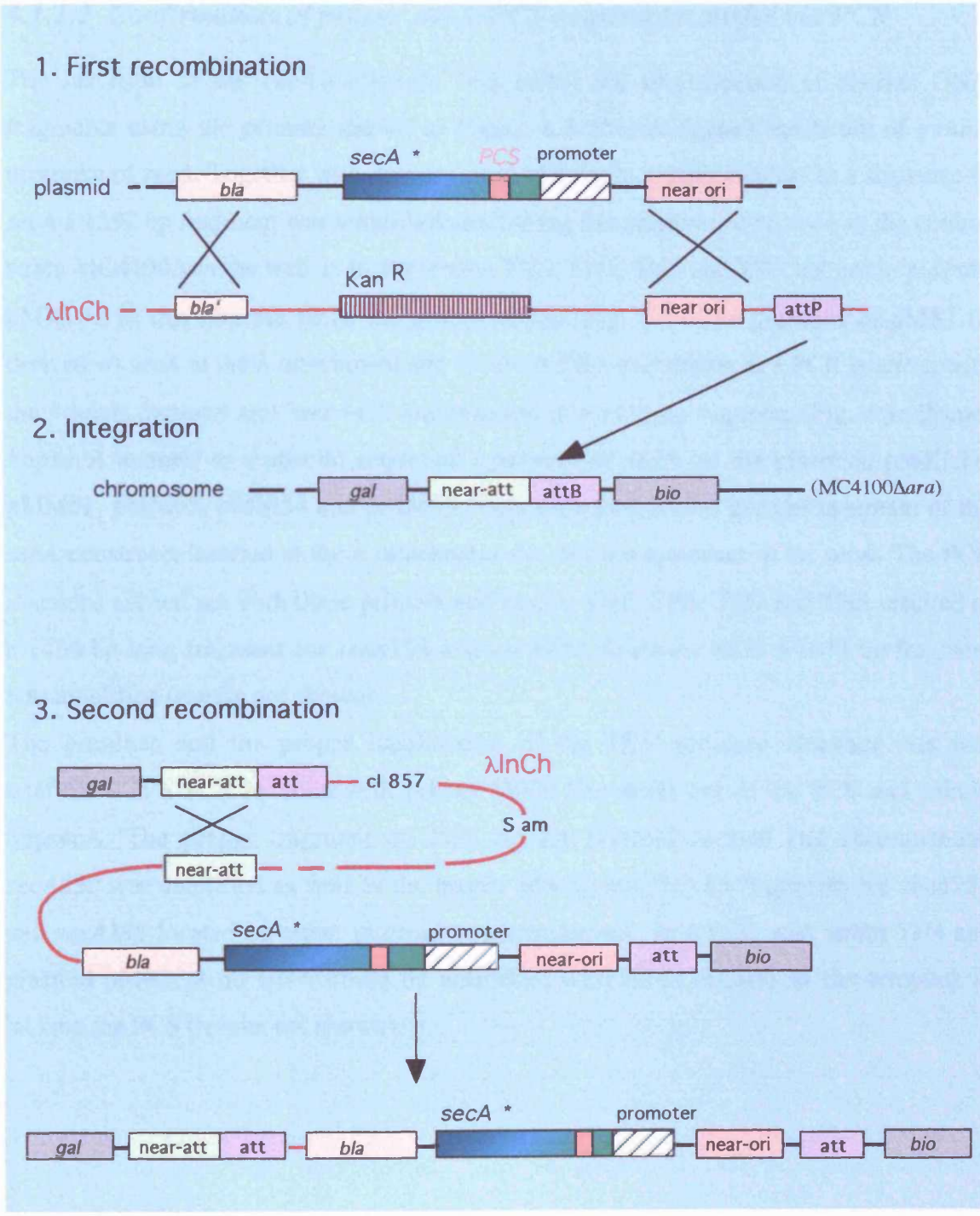


Fig. 4.2 Schematic view of the integration of *secA*-constructs into the *E. coli* chromosome via λInCh. Plasmid DNA and chromosomal DNA is shown in black, phage DNA in red. The cross represents a recombination event. Abbreviations: am = amber mutation, att = attachment site.

4.1.1.2 Confirmation of proper secA-PCS construct transfer via PCR

The insertion of the *secA*-constructs was tested via amplification of distinct DNA fragments using the primers shown in Figure 4.3. Primer 5gene*X* reads out of *geneX* upstream of *secA*. Together with primer 3*secA*452 that is complementary to a sequence in *secA* a 1592 bp fragment was amplified confirming the presence of wt *secA* in the control strain MC4100Δ*ara* as well as in the strains TH5, TH6, TH7 and TH8 but not in plasmid pMKL18 as this plasmid lacks the gene*X* region (Fig. 4.4). The presence of pMKL18 derived wt *secA* at the λ attachment site in strain TH4 was shown in a PCR reaction with the primers 5up*secA* and 3*secA*452 and resulted in a 1415 bp fragment (Fig. 4.5). Primer 5up*secA* anneals to a special sequence upstream of *secA* on the plasmids pMKL18, pMM51, pMM52, pMM54 and pMM55. This sequence is also present upstream of the *secA*-constructs inserted at the λ attachment site but not upstream of wt *secA*. The PCR reactions carried out with these primers and strains TH5, TH6, TH7 and TH8 resulted in a 1466 bp long fragment for *secA*195 and *secA*252. From *secA*830 a 1415 bp fragment was amplified (results not shown).

The presence and the proper localisation of the TEV protease cleavage site was confirmed in a PCR reaction with primer 3PCS that reads out of the PCS and primer 5upsecA. The proper fragment of 2598 bp for plasmid-derived and chromosomal *secA*830 was amplified as well as the proper 864 bp and 693 bp fragments for *secA*252 and *secA*195 located on either plasmid or chromosome. In a PCR with strain TH4 and plasmid pMKL18 no DNA could be amplified with these primers as the template is lacking the PCS (results not shown).

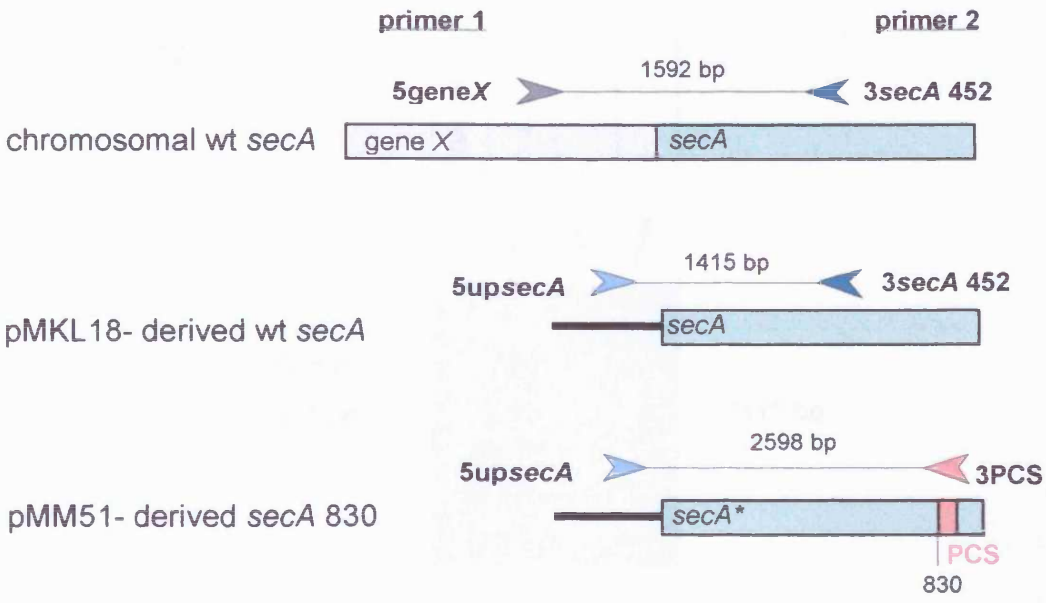


Fig. 4.3 Schematic illustration of primer annealing sequences in and around *secA*. Fragment sizes resulting from PCR reactions using the indicated primers are shown for chromosomal wt *secA* and for two plasmid-derived *secA*-constructs located at the λ attachment site (wt *secA* and *secA*830).

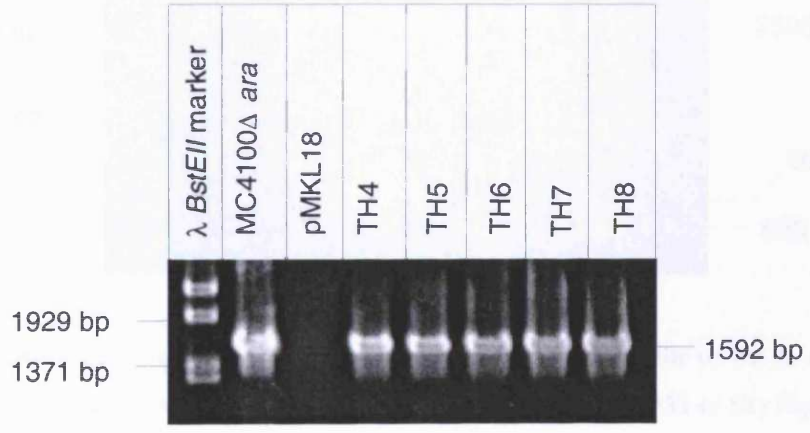


Fig. 4.4 Confirmation of the presence of chromosomal wt *secA* via PCR. PCR-products were separated on a 0.9% agarose gel. For amplifying DNA fragments from plasmid pMKL18 (wt *secA* without gene*X*) 1 µl template DNA was used in a 100 µl sample whereas a colony PCR was performed with strains MC4100Δ*ara*, TH4, TH5, TH6, TH7 and TH8 using primers 5gene*X* and 3*secA*452 and *taq* DNA polymerase.

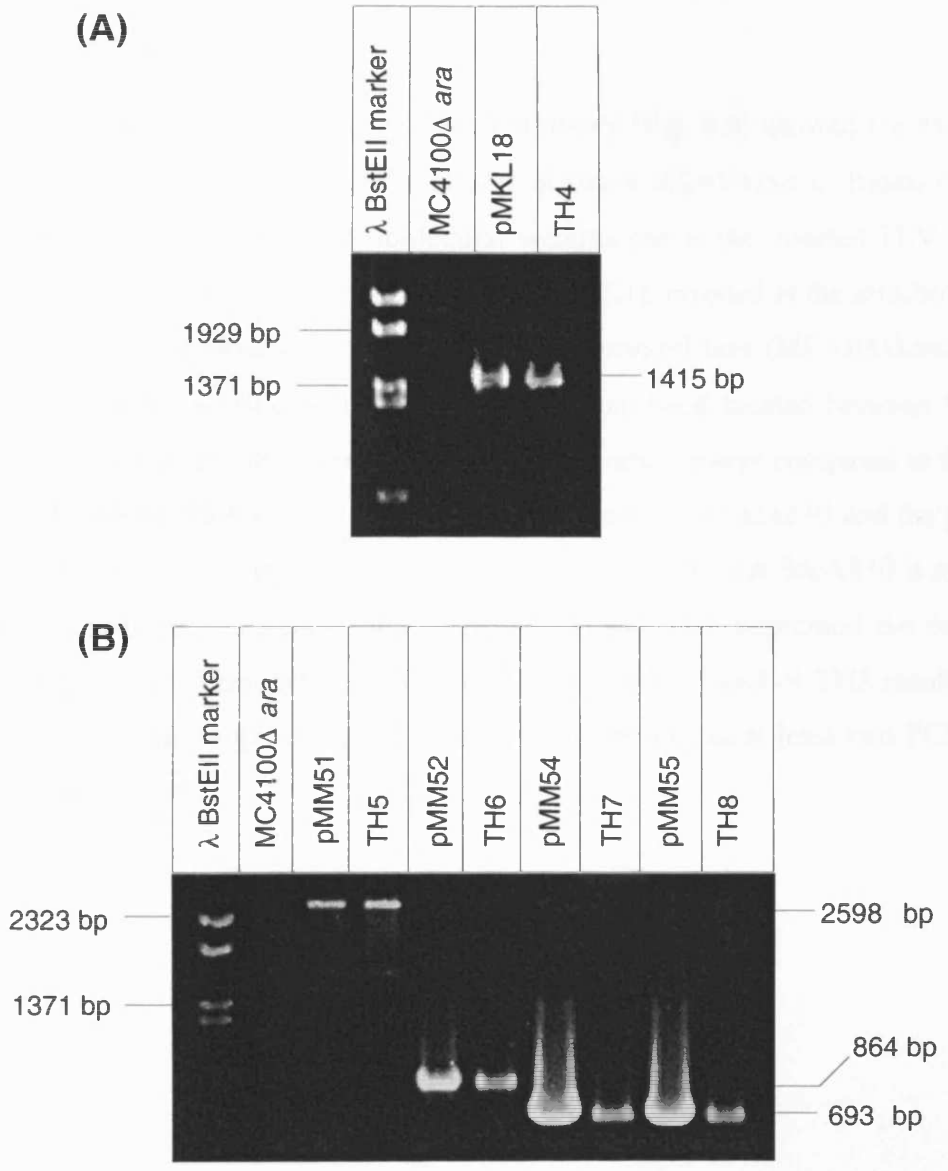


Fig. 4.5 Confirmation of proper *secA*-PCS insertion in the λ attachment site on the *E. coli* chromosome via PCR with *taq* DNA polymerase and (A) primers 5up*secA* and 3*secA*452 or (B) 5upSecA and 3PCS respectively. 1 µl template DNA from plasmids pMKL18 (wt *secA*), pMM51 (*secA*830), pMM52 (*secA*252), pMM54 (*secA*195) and pMM55 (*secA*195,2) was used in a 100 µl reaction. A colony PCR was performed with strains MC4100Δ*ara*, TH4 (wt *secA*), TH5 (*secA*830), TH6 (*secA*252), TH7 (*secA*195) and TH8 (*secA*195,2).

4.1.1.3 Expression of chromosomal wt secA and chromosomal secA-PCS constructs

Western blot analysis performed with α-*SecA* antibody (Fig. 4.6) showed the expression of wild-type SecA and of the SecA-constructs in strain MC4100Δ*ara*. Bands of SecA-constructs displayed slightly higher molecular weights due to the inserted TEV protease cleavage sites. Strain TH4 carries wt *secA* from pMKL18 inserted at the attachment site, therefore, the wt SecA band was stronger than in the control lane (MC4100Δ*ara*). Strain TH5 carrying the SecA830-construct showed only one band located between wt SecA and SecA-PCS bands and the expression level was slightly lower compared to the other constructs. However, PCR controls confirmed the insertion of *secA*830 and the presence of wt *secA* in this strain (chapter 4.1.1.2). It might be possible that SecA830 is not stable and a truncated form was visible on the blot. TH6 and TH7 expressed the constructs SecA252 and SecA195 respectively. The higher SecA-PCS band of TH8 resulted from the higher molecular weight of SecA195,2. This construct has at least two PCS cloned behind amino acid 195.

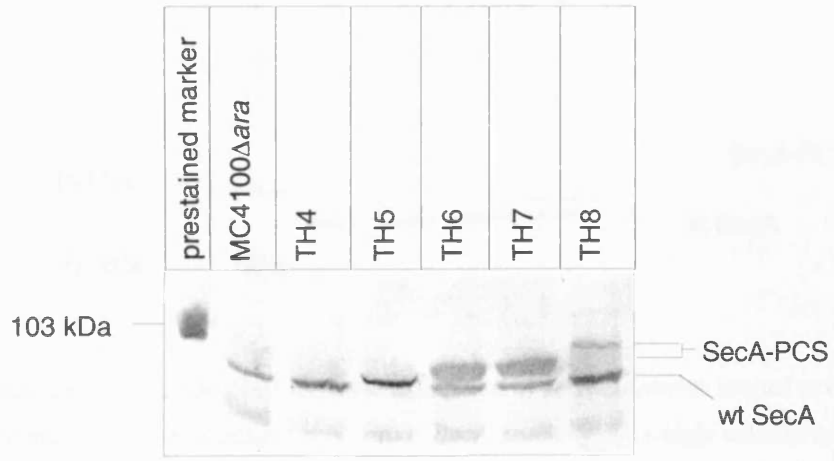


Fig. 4.6 Expression of chromosomal *secA* and expression of *secA*-constructs located at the attachment site. Western blot analysis with α-SecA antibody from a 7% SDS-PAGE. Samples were taken from overnight cultures in LB medium containing 25 µg/ml ampicillin. The culture of MC4100Δ*ara* was grown in LB medium only. TH4 expressed an additional wt *secA* located at the att site. The strains TH5, TH6, TH7 and TH8 expressed the chromosomal *secA*-constructs *secA*830, *secA*252, *secA*195 and *secA*195,2 respectively.

In the above strains the *secA*-PCS constructs are constitutively expressed due to the lack of the *lac* repressor. To test for regulatable expression under *lac* promoter control plasmid pTHS19 was cloned as a derivative of pVC19. This plasmid carries the $lacI^q$ gene from plasmid pCS19 and the tetracycline resistance gene as well as the origin of replication from pACYC184. Plasmid pVC19 was cut with the restriction enzymes *Mfe I* and *Blp I* to eliminate the 6x His tag resulting in pTHS19.

Strains TH6 and TH7 were transformed with plasmid pTHS19. Western blot analysis with α-SecA antibody is shown in Figure 4.7. Strains induced with IPTG for 3 hours expressed the SecA-PCS construct, whereas without induction no SecA-PCS band was visible. Chromosomal wt SecA was expressed under all conditions.

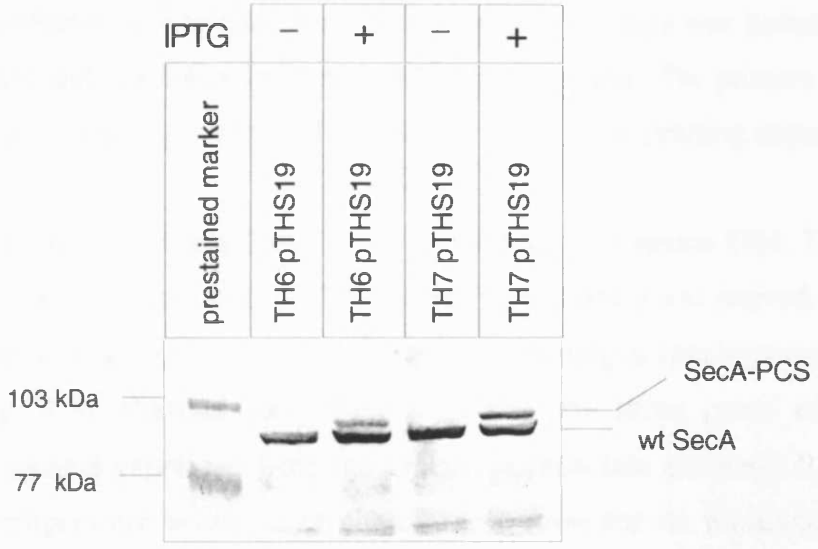


Fig. 4.7 Expression of chromosomal *secA* and expression of *secA*-constructs located at the attachment site. Western blot analysis of whole cell extracts with α-SecA antibody. Overnight cultures were grown in NZA medium containing 25 µg/ml ampicillin and 5 µg/ml tetracycline and diluted 1/100 in the same medium. One culture of each strain was induced with 100 µg/ml IPTG. Samples were taken after 3 hours of growth and loaded on a 7% SDS-PAGE.

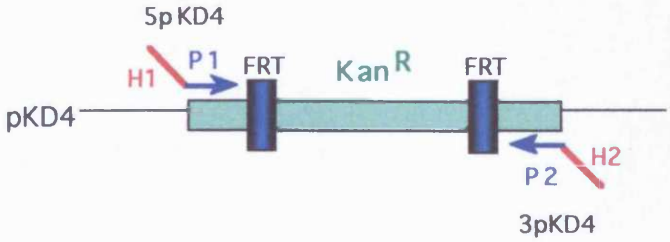
4.1.1.4 Elimination of chromosomal wt secA by using the λ Red recombinase system

Datsenko and Wanner recently described a method to directly disrupt chromosomal genes in *E. coli* by using primers with homologous sequences to the targeted gene (Datsenko and Wanner, 2000). Recombination events are performed by the λ Red recombinase that was shown to produce more enhanced rates of recombination of linear DNA fragments than *recBC sbcB* or *recD* mutants (Murphy, 1998). The λRed recombinase system consists of three genes. The product of the γ gene inhibits the host RecBCD exonuclease V so that the two other gene products, called Bet and Exo can perform the recombination. This method was used and adapted here to eliminate *secA* from the chromosome of the experimental strains.

The three steps of this method are illustrated in Figure 4.8. First, a 1.4 kb PCR product containing the kanamycin resistance gene flanked by FRT sites was generated by using primers 3pKD4 and 5pKD4 and plasmid pKD4 as a template. The primers include a 37-nt or 35- nt homology region (H1, H2) and a 18-nt or 21-nt priming sequence (P1, P2) respectively.

The purified PCR product was then electrotransformed into strains TH4, TH5, TH6 and TH7 carrying the low copy plasmid pKD46spec. This plasmid was derived from plasmid pKD46 by removing most of the *bla* gene and inserting a spectinomycin resistance cassette (Fig. 4.9). Plasmid pKD46spec encodes the three genes of the λ Red recombinase system expressed from the arabinose inducible promoter P_{araBAD}. It also contains a temperature sensitive origin of replication for its elimination after the recombination event. Competent cells for electrotransformation were grown in the presence of arabinose to induce the recombinase system and kanamycin resistant transformants were selected at 37°C or 42°C and tested for the loss of the recombinase helper plasmid. Strain TH6 was the first candidate that produced kanamycin resistant and spectinomycin sensitive transformants. Isolated transformants were tested by PCR (chapter 4.1.1.6) and the resulting strain carrying *secA252* at the attachment site and the Kan^R-FRT fragment instead of the wt *secA* was named TH6K.

1. PCR amplification of FRT- flanked kanamycin resistance gene



2. Recombination between homology extensions and chromosomal sequences in a strain expressing λ Red recombinase

3. Selection of kanamycin-resistant transformants

Fig. 4.8 Illustration of the wt *secA* elimination. Primer 5pKD4 and 3pKD4 include the homology regions adjacent to *secA* H1 and H2 respectively as well as the priming sequences P1 and P2 for plasmid pKD4. Abbreviation: FRT = FLP recognition target. (Adapted from Datsenko and Wanner, 2000)

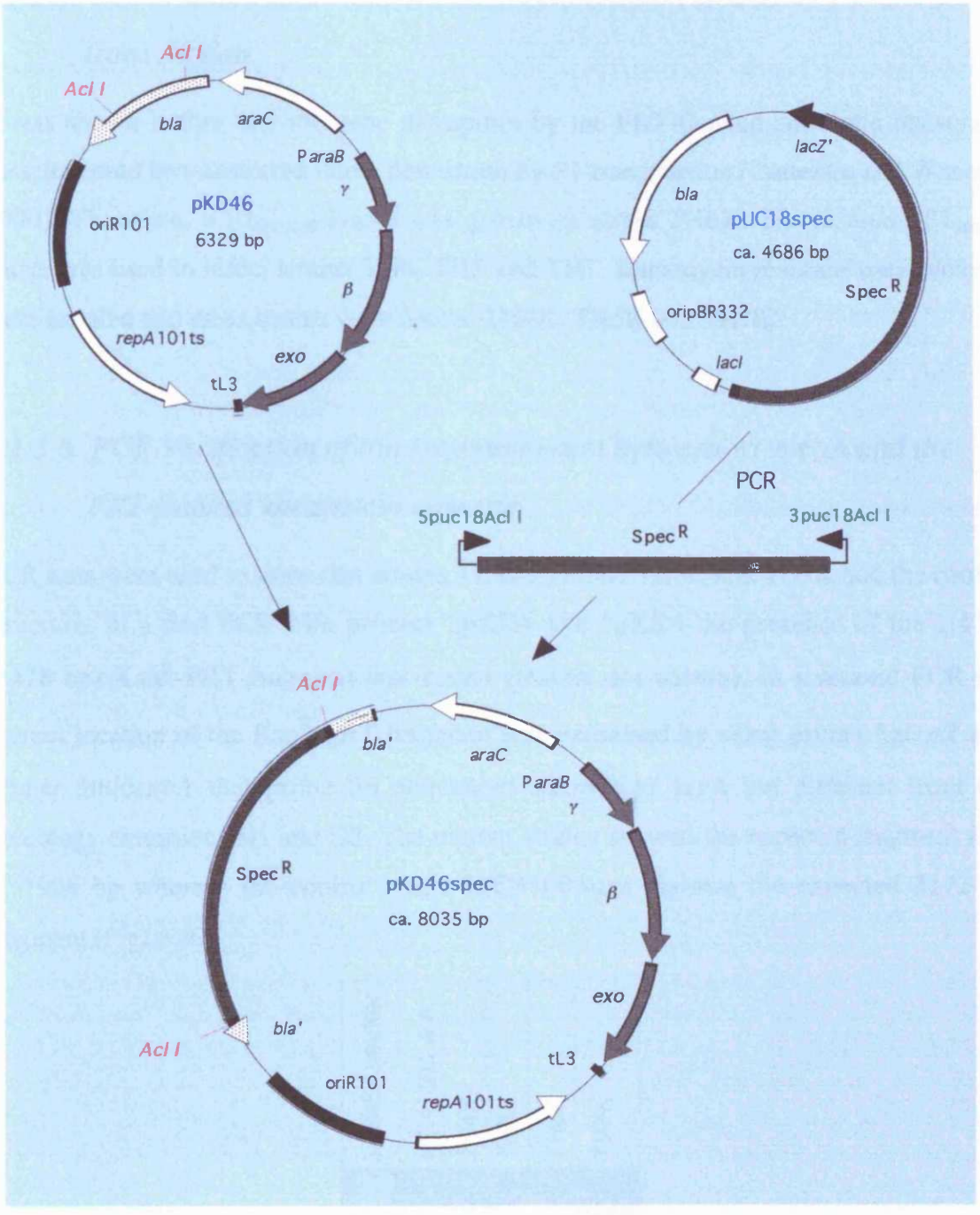


Fig. 4.9 Cloning of pKD46spec. The PCR-amplified spectinomycin resistance cassette from pUC18spec was cloned into the pKD46 vector fragment via ligation of the *AclI* restriction site. The restriction site is shown in red, primer containing the *AclI* site in green. Sequences of primers are stated in Table 3.12 in chapter 3.6.6.3.

4.1.1.5 Construction of further wt secA-elimination strains by P1-transduction

It was shown before that the gene disruption by the FRT-flanked antibiotic resistance cassette could be transferred into a new strain by P1-transduction (Datsenko and Wanner, 2000). Therefore, a $P1_{MC4100}$-lysate was grown on strain TH6K. The resultant $P1_{TH6K}$-lysate was used to infect strains TH4, TH5 and TH7. Kanamycin resistant transductants were isolated and these strains were named TH4K, TH5K and TH7K.

4.1.1.6 PCR Verification of the recombination between wt secA and the FRT-flanked kanamycin cassette

PCR tests were used to show that strains TH4K, TH5K, TH6K and TH7K had the correct structure. In a first PCR with primers 3pKD4 and 5pKD4 the presence of the 1.4 kb (1478 bp) Kan^R-FRT fragment was tested (results not shown). In a second PCR the correct location of the Kan^R-FRT fragment was examined by using primer 5gene*X* and primer 3into*secA* that prime for sequences outside of *secA* but different from the homology extensions H1 and H2. The mutant strains showed the expected fragment size of 1906 bp whereas the control strain MC4100Δ*ara* showed the expected 3172 bp fragment (Fig. 4.10).

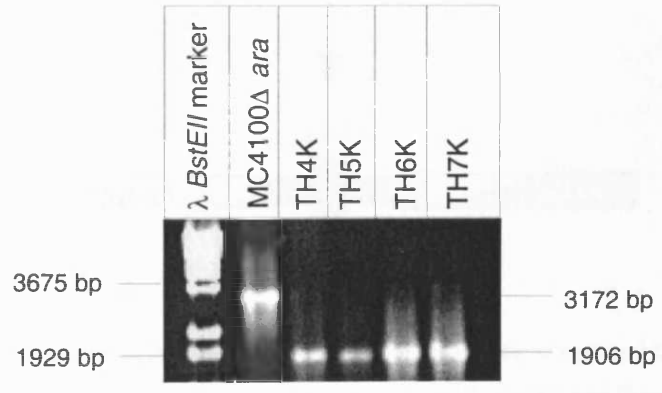


Fig. 4.10 Confirmation of the correct location of the Kan^R-FRT fragment in the above strains via PCR. PCR-products were separated on a 0.9% agarose gel. MC4100Δ*ara* was used as a control.

4.1.1.7 Elimination of the FRT-flanked kanamycin cassette

The kanamycin resistance cassette was eliminated by FLP recombinase promoted recombination between the FRT sites (Fig. 4.11). Strains TH4K, TH5K, TH6K and TH7K containing the FRT flanked kanamycin cassette were transformed by electroporation with the FLP recombinase helper plasmid pCP20 that contains a temperature sensitive replicon and a chloramphenicol resistance gene. After purification at 42°C the majority of the transformants showed kanamycin and chloramphenicol sensitivity. Strains with the *secA*-deletion were named TH24, TH25, TH26 and TH27 respectively. The remaining scar at the former location of *secA* includes a FRT site and the pKD4 priming sites P1 and P2 (Fig. 4.12). This scar also has stop codons in all six reading frames and a ribosome binding site. The original P2 sequence as described by Datsenko and Wanner also contained a start codon, so that pKD4 can be used to create nonpolar gene disruptions. Here, the P2 sequence is lacking 2 nucleotides to enhance PCR efficiency which destroys the start codon. However, the option to start transcription from this site is of no importance here.

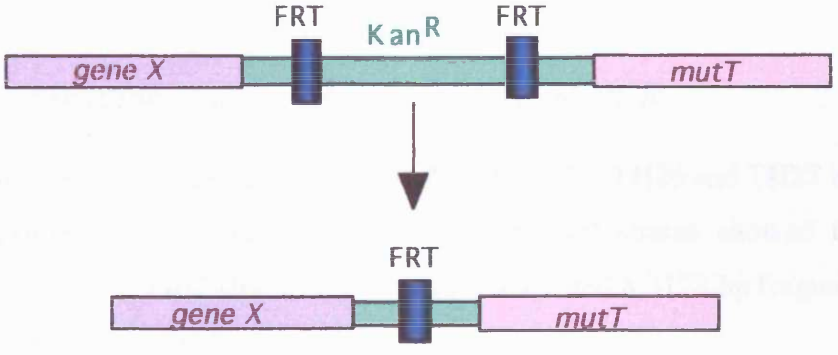
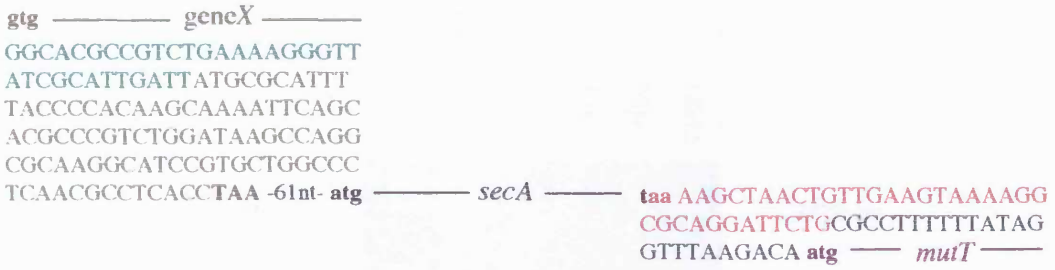


Fig. 4.11 Schematic view of the elimination of the kanamycin cassette via recombination between the FRT sites in strains expressing FLP recombinase. (Adapted from Datsenko and Wanner, 2000).

A. Template sequence for homology extensions of primers 3pKD4 and 5pKD4



B. Predicted sequence after FLP- mediated excision of kanamycin resistance

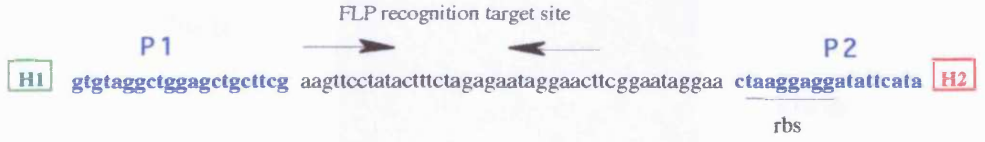


Fig. 4.12 Relevant sequences before and after the elimination of *secA*. (A) Illustration of the homology sequences H1 and H2 and adjacent regions on the chromosome. (B) Sequence on the chromosome remaining after the excision of the FRT-flanked kanamycin cassette. Start and stop codons are written in bold letters. The inverted repeats of the FRT site are marked with arrows. H1 is shown in green, H2 in red and the priming sites P1 and P2 are shown in blue.

4.1.1.8 Confirmation of delta-wt secA strains via PCR

The loss of the kanamycin cassette in strains TH24, TH25, TH26 and TH27 was tested by PCR with primers 5gene*X* and 3intosecA. All mutant strains showed the expected fragment of 513 bp. Control strain MC4100Δ*ara* delivered a 3172 bp fragment in a PCR with the same primers (Fig. 4.13).

In addition test-PCRs were performed with primers 5gene*X* and 3*secA*452 that resulted in a 1592 bp fragment when MC4100Δ*ara* was used. As expected no fragment was amplified in a PCR with these primers and the mutant strains (results not shown). The correct structure of the *secA*-PCS construct located at the attachment site was again verified with the PCRs described in chapter 4.1.1.2 (results not shown).

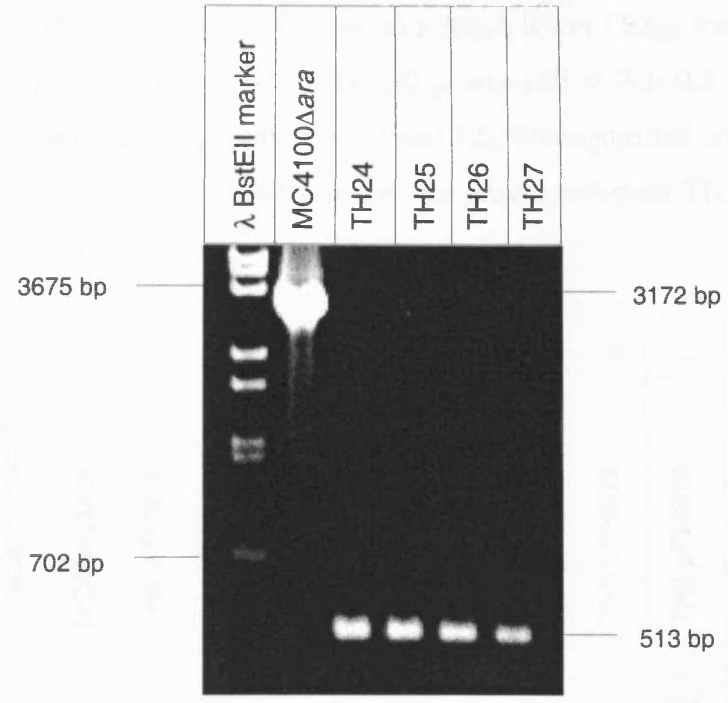


Fig. 4.13 Confirmation of the loss of the Kan^R-FRT fragment in the above strains via PCR. PCR-products were separated on a 0.9% agarose gel. MC4100Δ*ara* was used as a control.

4.1.1.9 Expression of chromosomal secA-PCS constructs

Strains TH24, TH25, TH26 and TH27 that are all lacking wt *secA* carry no *lac* repressor so that the SecA-PCS constructs are constitutivly expressed. To test the regulated expression from the *lac* promoter the strains were transformed with plasmid pTHS19 carrying the $lacI^q$ gene. The Western blot analysis with α-SecA antibody showed that in the presence of the inducer IPTG (final concentration of 100 µM) the *secA*-PCS constructs were expressed in all strains (Fig. 4.15). TH24 expressed wt SecA behind P_{lac} whereas, as expected, the SecA bands resulting from strains TH25, TH26 and TH27 were slightly higher due to the additional molecular weight caused by the presence of the PCS. The absence of a wt SecA band in these strains confirmed the successful elimination of the gene. Surprisingly, only TH25 showed less expression of SecA-PCS (SecA830) in the absence of IPTG. All other strains expressed the SecA-construct without induction. TH25

pTHS19 also was the only strain that grew to a much lower OD_{600} without the inducer. After 7 hours of growth without IPTG the OD_{600} was still at 0.2- 0.3 whereas all other cultures were grown to an OD_{600} between 2.3 and 3.0. When purified on NZA agar plates without IPTG TH25 pTHS19 showed almost no growth whereas TH24 pTHS19 grew well without IPTG (Fig. 4.16).

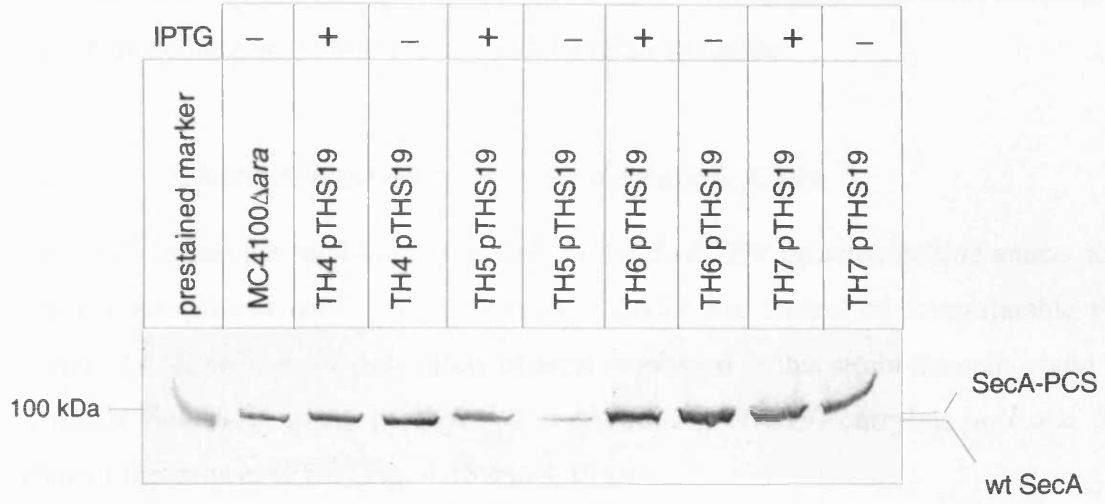


Fig. 4.15 Expression of chromosomal *wt secA* and *secA*-constructs located at the attachment site behind the *lac* promoter. Western blot analysis with α-SecA antibody from a 10% SDS-PAGE. Overnight cultures were grown in NZA medium with 5 µg/ml tetracycline, 25 µg/ml ampicillin and 100 µM IPTG. Cultures were washed 3 times and diluted 1/100 in the same medium without IPTG or 100 µM IPTG respectively. Samples were taken after 7 hours of growth.

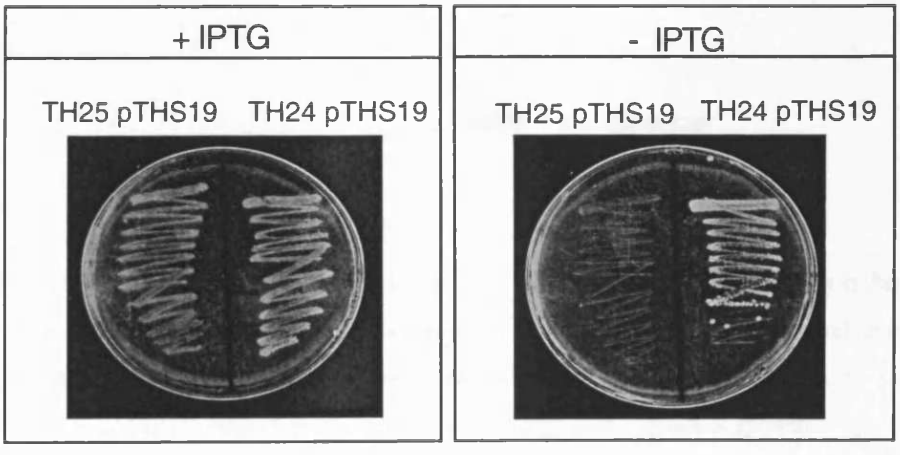


Fig. 4.16 Growth on NZA agar plates containing 5 µg/ml tetracycline 25 µg/ml ampicillin and no IPTG or 100 µM IPTG respectively. Plates were incubated at 37°C overnight.

These results indicate that during the elimination process of wt *secA* the regulation of the P_{lac} promoter in strains TH24, TH26 and TH27 was damaged or inhibited. This phenomenon was observed in most cases even directly after the transformation of the still regulatable original strains TH4, TH5, TH6 and TH7 with pKD46spec. Although the elimination of wt *secA* was performed several times, with or without the pTHS19 present in the strains and with P1 transduction of the Kan^R-FRT fragment from different strains, TH25 remained the only strain with a regulatable *lac* promoter.

4.1.1.10 Characterisation of a SecA-depletion strain

Strain TH25 carries the *secA*-construct with the PCS of TEV protease behind amino acid 830 at the attachment site. The expression is under the control of a regulatable P_{lac} promoter. As *secA*830 *is* the only allele of *secA* expressed in this strain the cells could be depleted of SecA830 in the presence of a plasmid (pTHS19) carrying *lacI* and the absence of the inducer IPTG (Fig. 4.15 and 4.17).

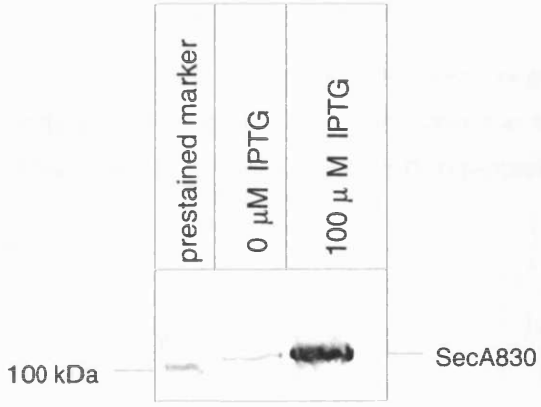


Fig. 4.17 Expression of *secA830* in strain TH25 pTHS19. Western blot analysis with α-SecA antibody from a 10% SDS-PAGE. Overnight cultures were grown in NZA medium with 5 µg/ml tetracycline, 25 µg/ml ampicillin and 100 µM IPTG. Cultures were washed 3 times and diluted 1/100 in the same medium without IPTG or 100 µM IPTG respectively. Samples were taken after 7 hours of growth.

When no IPTG was supplied growth of TH25 pTHS19 stopped at an OD_{600} of about 0.3 (Fig. 4.18). The cells grew to higher optical densities dependent on the IPTG concentration in the culture. With a concentration of 100 mM IPTG the culture showed the steepest growth curve and reached an OD_{600} of 2.8 in 7 hours.

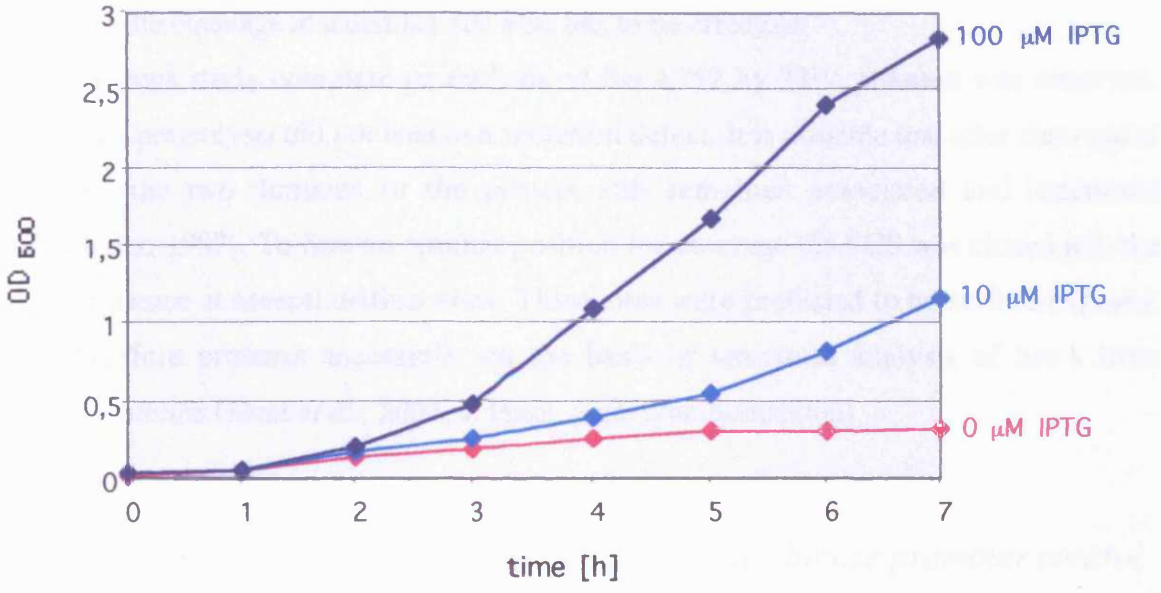


Fig. 4.18 Growth curve of strain TH25 pTHS19. An overnight culture was grown in NZA medium with 5 µg/ml tetracycline, 25 µg/ml ampicillin and 100 µM IPTG. The culture was washed 3 times and diluted in the same medium without IPTG or 10 µM IPTG and 100 µM IPTG respectively.

4.1.2 Introducing and characterisation of new TEV protease cleavage sites in SecA

The position of the PCS in SecA is crucial for a successful proteolysis of the protein. The cleavage site has to be accessible for the protease. Regarding the inactivation of SecA function, the cleavage at a distinct site also has to be effective.
In a previous study complete proteolysis of SecA252 by TEV protease was observed. However, proteolysis did not lead to a secretion defect. It is possible that after cleavage at this site the two domains of the protein still remained associated and functional (Mondigler, 1997). To find an optimal position for cleavage the PCS was cloned into the *secA* sequence at several distinct sites. These sites were predicted to be surface exposed, and therefore protease accessible, on the basis of structural analysis of SecA from *Bacillus subtilis* (Hunt *et al.*, 2002; J. Hunt, pers. communication).

4.1.2.1 Cloning of secA-PCS constructs under arabinose promoter control

To clone the PCS at distinct positions in the *secA* sequence the plasmid pEDIE-2 was used. This plasmid carries a PCS between the arabinose promoter P_{araBAD} (Guzman *et al.*, 1995) and the gene *uidA*, which is out of reading frame. *uidA* encodes the enzyme β-glucuronidase. This enzyme cleaves the substrate X-GLUC, leading to blue colonies on agar plates containing this substrate. The cloning strategy is shown in Figure 4.19. In a first PCR with the primers 5*secA* and 3*secA*229, 3*secA*296 or 3*secA*518 respectively, the N-terminal fragment of SecA was amplified using plasmid pMF8 as a template. The primers contain restriction sites for the endonucleases *Kpn I* and *Xba I* and the PCR product was cloned via those sites into pEDIE-2 between the promoter P_{araBAD} and the PCS. As a result the PCS was localised behind the amino acids 229, 296 and 518 of SecA and the reporter gene *uidA* was shifted into the proper reading frame. The strain EZ4 with a deleted *uidA* was transformed with the ligation reaction. Cells that had the N-terminal *secA* fragment properly inserted expressed active β-glucuronidase. Blue cells for all three constructs were screened on agar plates with 0.1% arabinose, ampicillin and the substrate

X-GLUC. Without the inducer arabinose in the plates only white colonies were formed. After purification and a repeated test for active β-glucuronidase on X-GLUC agar plates the DNA was isolated and a control restriction digestion was performed with *Kpn I* and *Xba I*. The correct fragment length was obtained for all three constructs.

In a second step the *uidA* gene in pEDIE-2 was cut out via *Not I* restriction sites and the remaining C-terminal fragments of *secA* were amplified in a PCR using the primers 3*secA* and 5*secA*230, 5*secA*297 or 5*secA*519 respectively. The primers were designed with *Not I* restriction sites to clone the C-terminal *secA* fragment into pEDIE-2 behind the PCS. The *Not I* dependent ligation and the *Not I* control digestion reactions were performed by the company GATC AG (Konstanz, Germany). The resulting plasmids containing the *secA*-constructs *secA*229, *secA*296 and *secA*518 under arabinose promoter control were termed pSecA229, pSecA296 and pSecA518. Strain EZ4 transformed with these plasmids formed white colonies on agar plates containing X-GLUC.

Further *secA*-PCS constructs were cloned by GATC AG (Konstanz, Germany) as a part of the Diploma work of Charlotte Conz (Conz, 2000). The PCS was cloned as described above behind the amino acids 60, 295, 471 and 497 of the SecA protein and the plasmids were termed pSecA60, pSecA295, pSecA471 and pSecA497 respectively.

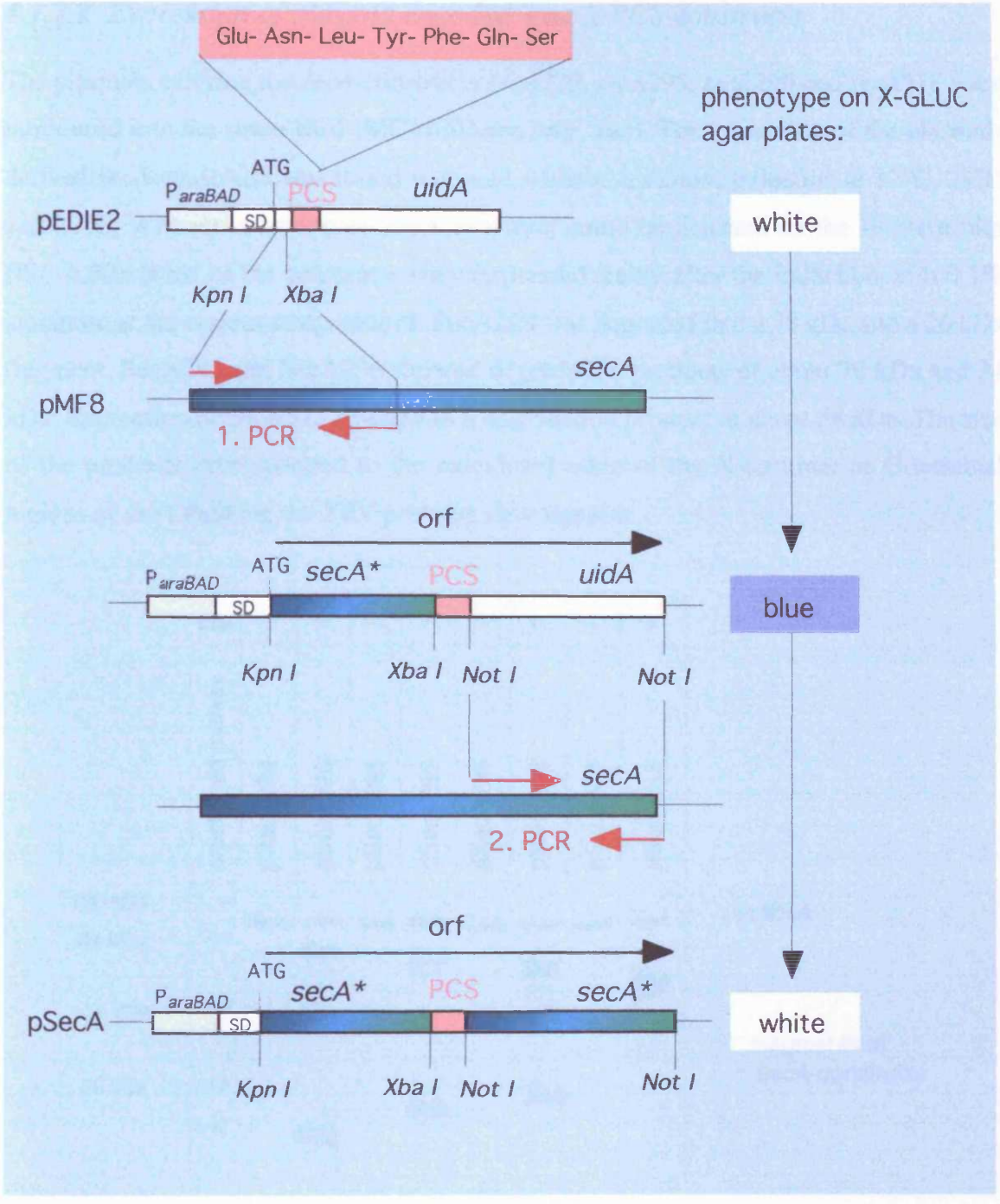


Fig. 4.19 Schematic representation of the cloning strategy to introduce the PCS at distinct positions in the sequence of *secA*. The plasmid pEDIE-2 carries the arabinose promoter, the PCS and the reporter gene *uidA*. The plasmid pMF8 served as a template to amplify N-terminal and C-terminal fragments of *secA*. These fragments were cloned into pEDIE-2 via the indicated restriction sites. Abbreviations: orf = open reading frame, SD = Shine-Dalgarno sequence, PCS = protease cleavage site.

4.1.2.2 *Expression of plasmid encoded secA-PCS constructs*

The plasmids carrying the *secA*-constructs *secA*229, *secA*295, *secA*296 and *secA*518 were introduced into the strain CC4 (MC4100Δ*ara* Δ*tig*::*kan*). The expression of the plasmid-derived *secA*-constructs was tested with and without arabinose induction at 37°C, 28°C and 20°C. Without induction no *secA*-construct could be detected on the Western blot (Fig. 4.20). None of the constructs were expressed stably after the induction with 0.1% arabinose at the various temperatures. SecA229 was degraded into a 78 kDa and a 26 kDa fragment. SecA295 and SecA296 showed degradation products of about 70 kDa and 34 kDa. Expression of SecA518 resulted in a degradation product of about 58 kDa. The size of the products corresponded to the calculated sizes of the N-terminal or C-terminal regions of *secA* flanking the TEV protease cleavage site.

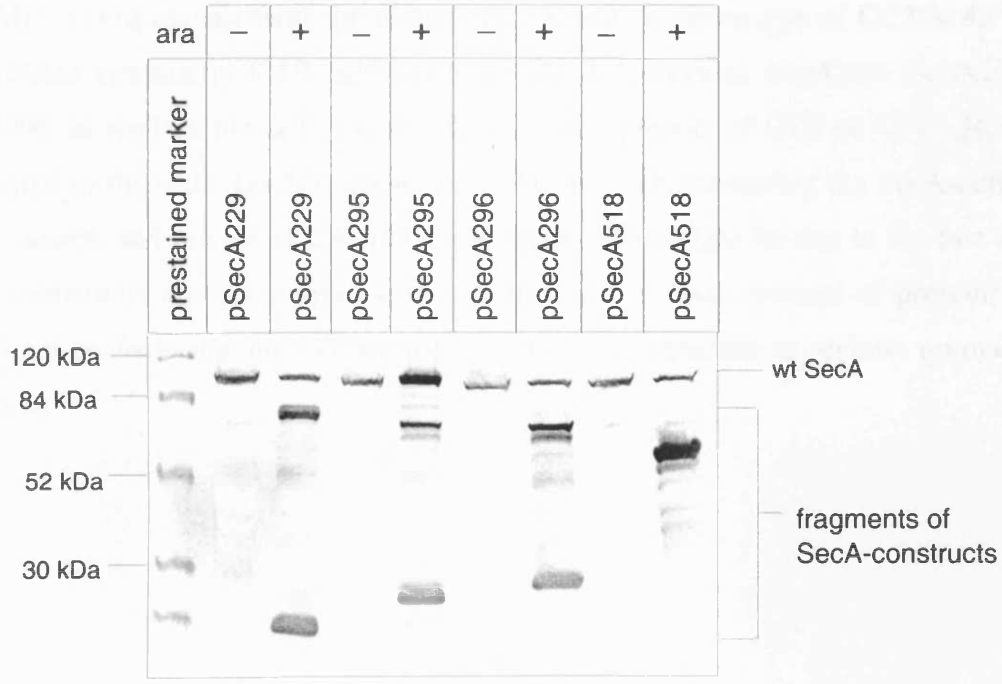


Fig. 4.20 Expression of plasmid derived *secA*-constructs in strain CC4 at 37°C. Western blot analysis with α-SecA antibody from a 10% SDS-polyacrylamide gel. Samples were taken from 1/100 diluted overnight cultures after 3 hours of growth in NZA ampicillin medium without or with 0.1% arabinose. Strain CC4 also expressed chromosomal wt *secA*

4.1.2.3 Complementation test for functional secA-PCS constructs

The ability of the SecA-constructs to complement a temperature sensitive mutant of *secA* was tested to find out whether the introduction of the PCS itself had a negative effect on the function of SecA. Strain CC3 contains the allele *secA*51(Ts) which leads to secretion defects at elevated temperatures. This strain is able to grow at 28°C but not at 42°C (Fig. 4.21). Strain CC3 was transformed with the plasmids pMF8, pSecA60, pSecA229, pSecA295, pSecA296, pSecA518, pSecA471 and pSecA497. The transformants were purified at 28°C on NZA ampicillin agar plates and showed normal growth. Transformants were then purified and incubated at 42°C to test whether the introduced SecA-construct could complement the temperature sensitive SecA and enable strain CC3 to grow at high temperature. The result of the complementation test is shown in Figure 4.22. Without induction of the *secA*-constructs with arabinose only the wt SecA encoded on pMF8 could complement the temperature sensitive phenotype of CC3 at 42°C. On agar plates containing 0.1% arabinose the SecA-constructs SecA229, SecA295 and SecA296 as well as partially SecA518 facilitated growth of CC3 at 42°C. However, compared to the cells transformed with pMF8, the cells expressing the SecA-constructs grew slower, and in case of SecA518 less dense. This might be due to the fact that all SecA-constructs are not stably expressed and only a small amount of protein, which might not be detectable on a Western blot, retains the structure to perform normal SecA function.

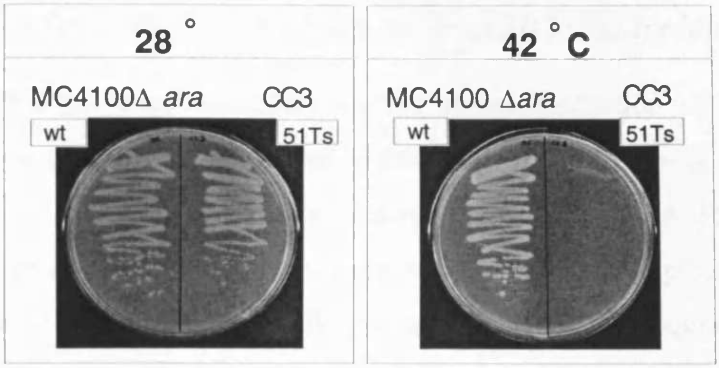


Fig. 4.21 Growth of strains MC4100 Δara and CC3 on NZA agar plates at 28°C and 42°C. The different *secA* alleles are indicated in small squares.

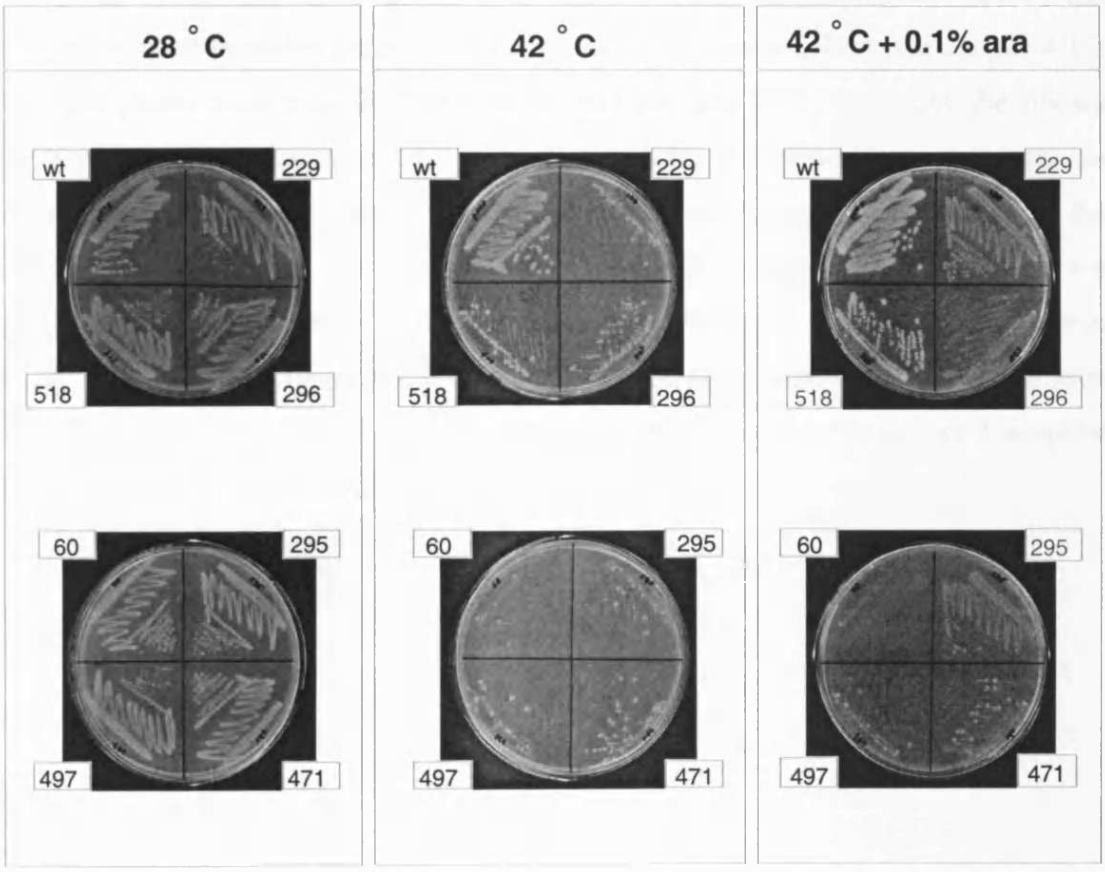


Fig. 4.22 Complementation of SecA51(Ts) in strain CC3. Strain CC3 was transformed with the plasmids pMF8, pSecA60, pSecA229, pSecA295, pSecA296, pSecA518, pSecA471 and pSecA497. NZA ampicillin agar plates with or without 0.1% arabinose were incubated over night at 28°C or 42°C. The different *secA* alleles are indicated in small squares.

4.1.2.4 Effect of SecA-PCS proteolysis on SecA51(Ts) complementation

The effect of TEV protease induction on the complementation of the temperature sensitive phenotype of CC3 was examined in this experiment. CC3 was transformed with pMF8 encoding wt *secA* as a control or plasmids pSec229, pSecA295 and pSecA296 respectively. In addition, each strain was transformed with plasmid pCC6 that carries the gene encoding the 27 kDa NIa-Pro (TEV protease) fused to the sequence encoding the first 144 amino acids of trigger factor. The short trigger factor fragment localises the TEV protease at the ribosome (Conz, 2000). The TEV protease-trigger factor fusion is under the control of the *tac* promoter.

At 28°C all transformed CC3 strains grew well, either without or with simultaneous induction of the TEV protease (Fig. 4.23). At 42°C the arabinose induced SecA-constructs complemented the *secA*51(Ts) mutation as shown before (see chapter 4.1.2.3). On agar plates containing 0.1% arabinose and 100 µM IPTG to induce the ribosomal TEV protease no growth of CC3 transformed with the plasmids carrying the *secA*-constructs was observed. This result might indicate that although expression of the SecA-constructs on a Western blot was not stable, probably a small amount that might not be detectable on a blot remains stable and functional. Probably these remaining functional SecA-constructs are a target of TEV-protease and cleavage prevented further cell growth. However, as also strain CC3 containing wt *secA* grew slower at the elevated temperature this conclusion is not altogether clear.

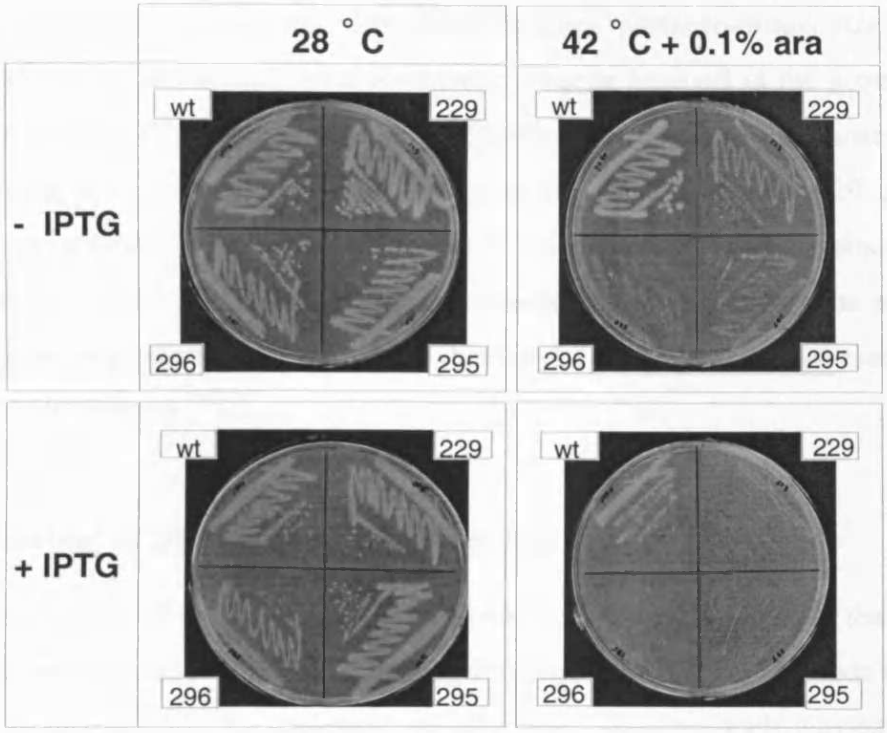


Fig. 4.23 Effect of TEV protease induction on SecA51(Ts) complementation. Strain CC3 pCC6 carrying either plasmid pMF8, pSecA229, pSecA295 or pSecA296 was grown on NZA agar plates containing 100 µg/ml ampicillin, 5 µg/ml tetracycline and no or 0.1% arabinose at 28°C and 42°C. Ribosomal TEV protease was induced on plates containing 100 µM IPTG. The different *secA* alleles are indicated in small squares.

4.1.2.5 Expression of secA-PCS constructs in protease minus strains

During the cloning-process of plasmids pSecA229, pSecA295, pSecA296 and pSecA518 (4.1.2.1) a sequence encoding 12 amino acids remained between the end of the N-terminal *secA* fragment and the sequence encoding the TEV-protease cleavage site. Apart from the two N-terminal amino acids of this sequence that form the restriction site *Xba I,* the rest of the sequence is insignificant. However, it is possible that these amino acids contributed to an unstable conformation of the protein or form a recognition site for one of the numerous cellular proteases.

To test whether the sequence around the PCS is recognised by cellular proteases the expression of the *secA*-constructs was tested in some protease-minus strains. Strain RU105 containing a chloramphenicol resistance cassette inserted in the gene encoding the cellular protease ClpP, and strain AFF220 carrying transposon Tn*10* inserted in the gene enconding the Lon protease were transformed with plasmids pSecA229, pSecA295, pSecA296 and pSecA518. Induction of the *secA*-constructs with 0.1% arabinose at 28°C did not result in a stable expression of the constructs. On a Western blot the same bands of the degradation products were visible as when *secA*-constructs were expressed in strain CC4 (results not shown).

4.1.2.6 Cloning of shortened secA-PCS constructs

To test if expression of the *secA*-constructs would be more stable without the additional amino acids between the N-terminal *secA* fragment and the PCS, the plasmids were PCR-amplified. In this reaction the sequence encoding the 12 amino acids was omitted. The sequence and the cloning strategy is shown in Figure 4.24. The primers were designed with a *Sma I* restriction site at the ends (primer sequence see chapter 3.6.6.3). Primer 5tetSMA was used for extension in 5' to 3' direction in all plasmids. A primer (3*secA*xSMA) specific for the appropriate *secA*-construct was used for extension in 3' to 5' direction. Via religation of the *Sma I* sites after restriction digestion the shortened plasmids pTH12 (*secA*229s), pTH13 (*secA*295s), pTH14 (*secA*296s) and pTH15 (*secA*518s) were constructed.

To verify the correct structure of the plasmids strain MC4100Δ*ara* was transformed with the plasmids and a control digestion was carried out with *Sma I* restriction endonuclease after plasmid isolation. Additionally, the affected segments of the plasmids were sequenced. Cycle sequencing reactions were performed as described in chapter 3.6.7 with the stated sequencing primers. All 4 plasmids contained the correct DNA-sequence with only the *Sma I* site between the N-terminal *secA* fragment and the PCS sequence.

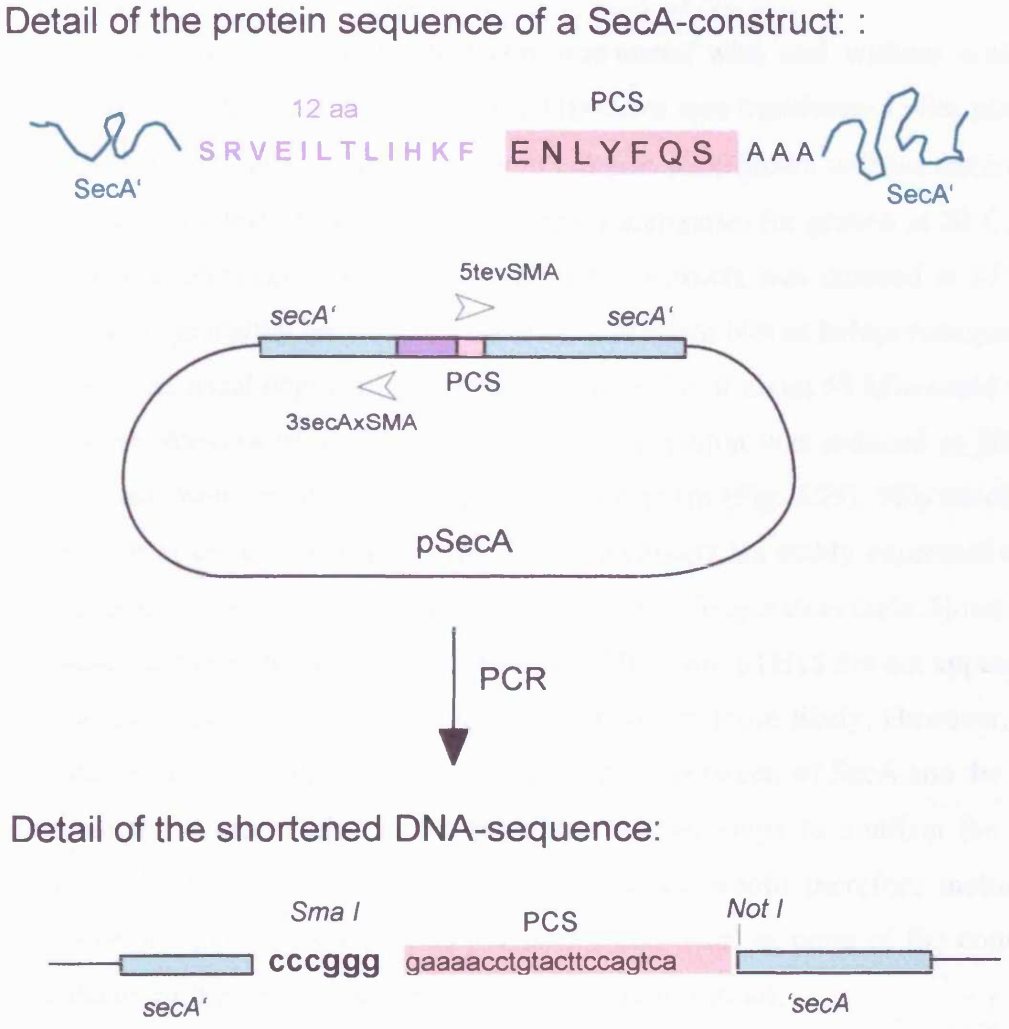


Fig. 4.24 Illustration of the cloning strategy to shorten the *secA*-constructs in plasmids pSecA229, pSecA295, pSecA296 and pSecA518. The 12 amino acid sequence (marked in lilac) was omitted in a PCR-amplification of the respective plasmid using *Sma I* site containing primers. The shortened *secA*-constructs carry only the newly introduced *Sma I* site in front of the PCS.

4.1.2.7 Expression of plasmid encoded shortened secA-PCS constructs

Expression of the shortened *secA*-constructs was tested with and without arabinose induction at various temperatures. Strain MC4100Δ*ara* was transformed with plasmids pTH12, pTH13, pTH14 and pTH15. Overnight cultures were grown without arabinose at 28°C and diluted into test medium (with or without arabinose) for growth at 20°C, 28°C or 37°C. When expression of the shortened *secA*-constructs was induced at 37°C the same pattern of degradation bands was visible on a Western blot as before (compare Fig. 4.20). At 28°C the usual degradation product of SecA518 of about 58 kDa could not be detected on the Western blot (Fig. 4.25). When expression was induced at 20°C no degradation bands were visible for SecA229s and SecA518s (Fig. 4.25). This result could either mean that at these temperatures the SecA-constructs are stably expressed or that putative degradation products are further degraded and no longer detectable. However, as the SecA bands in the arabinose induced lanes of pTH12 and pTH15 did not appear to be thicker as without induction the latter possibility is much more likely. However, as wt *secA* was also expressed in this experiment a distinction between wt SecA and the SecA-constructs on the Western blot is not possible. Further steps to confirm the stable expression of the two constructs at lower temperature would therefore include the elimination of wt *secA*. This step was not performed here, as none of the constructs seemed suitable for the construction of a SecA-inactivation strain.

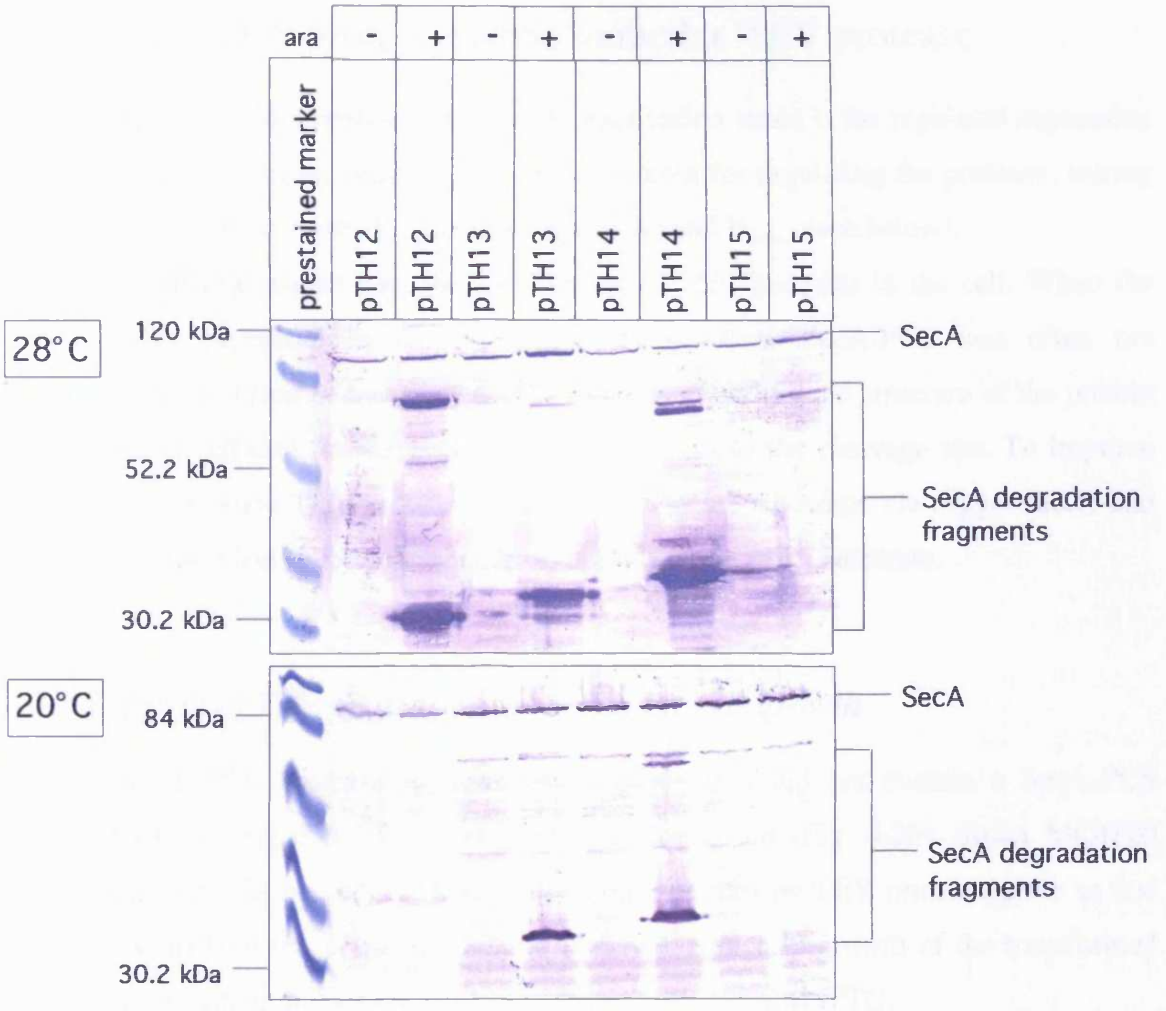


Fig. 4.25 Expression of shortened *secA*-constructs encoded on plasmids pTH12 (*secA*229s), pTH13 (*secA*295s), pTH14 (*secA*296s) and pTH15 (*secA*518) in strain MC4100Δ*ara*. Samples for a 10% SDS-polyacrylamide gel were taken from 1/100 diluted overnight cultures after 3 hours of growth at 28°C in NZA ampicillin medium without or with 0.1% arabinose, or after 8 hours of growth at 20°C in NZA ampicillin medium without or with 0.1% arabinose. Western blot ananlysis was performed with α-SecA antibody. Strain MC4100Δ*ara* also expressed chromosomal wt *secA*.

4.1.3 SecA-PCS proteolysis via inducible TEV protease

An important factor in constructing a SecA-inactivation strain is the regulated expression of TEV protease. Various promoters were considered for regulating the protease, among them the divergent promoter P_{malK} (see appendix A) and $P_{Ltet0\text{-}1}$ (see below).
Another significant aspect was the localisation of TEV protease in the cell. When the protease was expressed in the cytoplasm its substrate SecA-PCS was often not completely proteolysed or inactivated. The assembled and folded structure of the protein might make it difficult for the protease to gain access to the cleavage site. To improve proteolysis efficiency TEV protease was attached to the ribosome via trigger factor and brought in close vicinity to the nascent polypeptide chain of its substrate.

4.1.3.1 Effect of TEV protease expression on cell growth

Expression of TEV protease in a wild-type strain that did not contain a SecA-PCS construct had no negative effect on growth of the strain (Fig. 4.26). Strain MC4100 transformed with the plasmid pMM13 encoding cytoplasmic TEV protease grew as fast as MC4100 without the plasmid. There was no difference in growth of the transformed strain with or without induction of TEV protease with 100 µM IPTG.

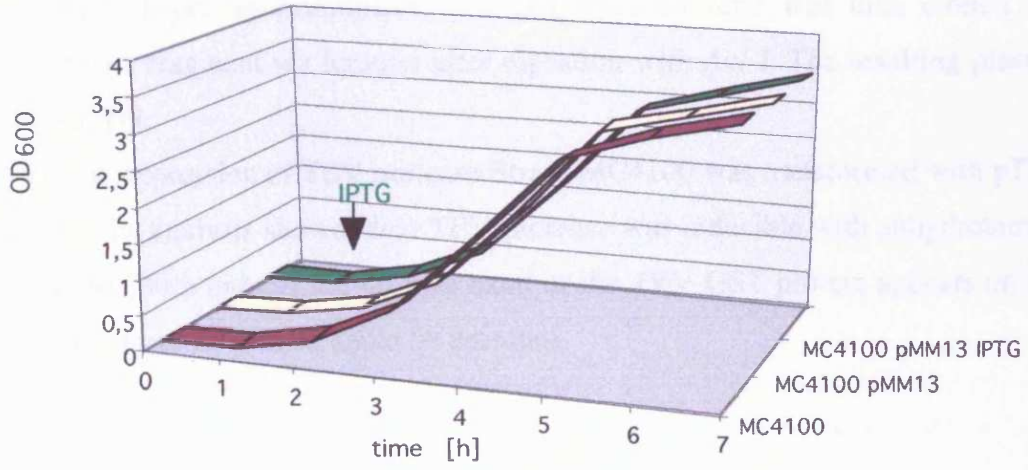


Fig. 4.26 Growth of strain MC4100 and MC4100 pMM13 (cytoplasmic TEV protease). Overnight cultures were diluted 1/100 in NZA medium with 5 µg/ml tetracycline for the transformed strains. TEV protease expression was induced after 1 hour of growth with 100 µM IPTG.

4.1.3.2 TEV protease under Ltet0-1 promoter control

A suitable promoter for the regulation of TEV protease was $P_{Ltet0-1}$ that consists of the phage lambda promoter P_L and sequences encoding operator 2 (tet02) of the Tn*10* tetracycline resistance operon (Lutz and Bujard, 1997). Not only was TEV protease regulated independently from its substrate, but expression was highly repressed by the repressor TetR in the absence of the inducer anhydrotetracycline. Promoter $P_{Ltet0-1}$ can be regulated up to a 5000-fold range when carried on plasmids like the pZS vectors with low copy origin pSC101 (Lutz and Bujard, 1997).

4.1.3.2.1 Cloning of plasmid pTH9 and expression of cytoplasmic TEV protease

On plasmid pZS*2tet0-1 the TEV protease is expressed under the control of the tight promoter $P_{Ltet0-1}$. This plasmid carries the low copy origin of replication pSC101 and a kanamycin resistance gene. The kanamycin resistance had to be exchanged against a spectinomycin resistance cassette as in some experiments the TEV protease was expressed in cells carrying a Δ*tig*::*kan* deletion. Part of the sequence of pZS*2tet0-1 was PCR-amplified with oligonucleotide primers containing the restriction site for the enzyme *Acl I* omitting most of the kanamycin resistance gene sequence (Fig. 4.27). A spectinomycin resistance cassette was PCR-amplified with *Acl I* site containing primers using pUC18spec as a template. The amplified cassette was then cloned into the pZS*2tet0-1 fragment via ligation after digestion with *Acl I*. The resulting plasmid was named pTH9.

To test the expression of TEV protease Strain MC4100 was transformed with pTH9. The Western blot analysis showed that TEV protease was inducible with anhydrotetracycline (Fig. 4.28). With inducer the 49 kDa band of the TEV-GST protein appears on the blot. Without inducer no protein could be detected.

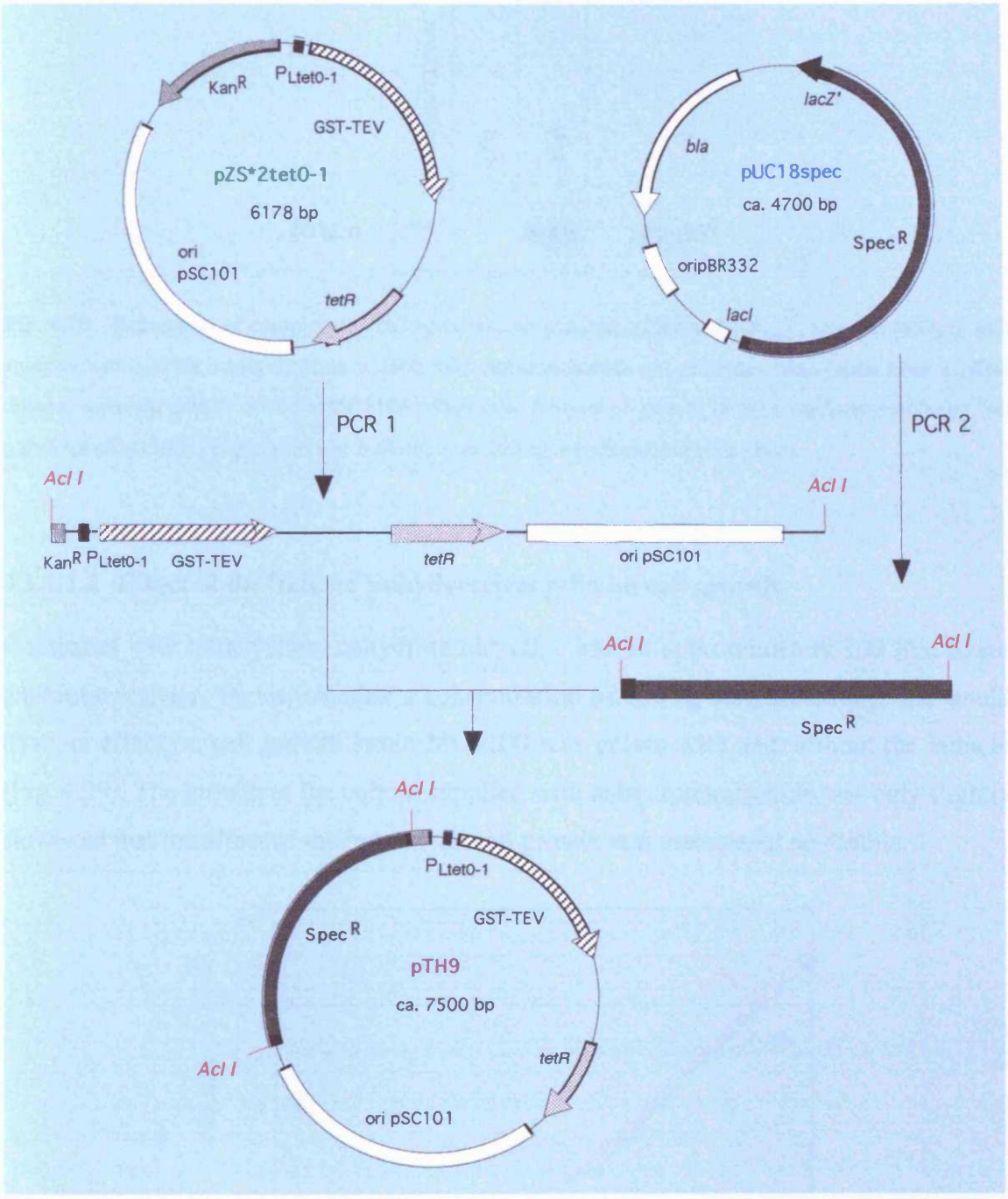


Fig. 4.27 Cloning of pTH9. Exchange of the kanamycin resistance gene against a spectinomycin resistance cassette in pZS*2tet0-1 via PCR and ligation of the *AclI* restriction sites resulting in plasmid pTH9. No precise DNA-sequence was available for pUC18spec. Therefore, only the approximate size of pUC18spec and plasmid pTH9 are given. The sequences of the used oligonucleotide primers are listed in Table 3.8 in chapter 3.6.6.3

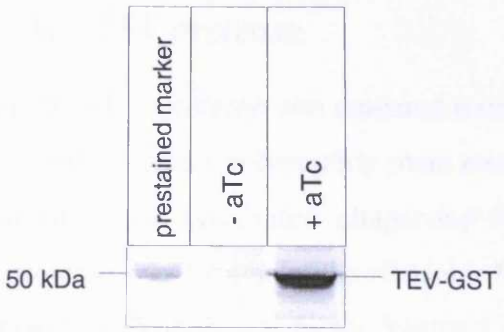


Fig. 4.28 Expression of cytoplasmic TEV protease on plasmid pTH9 under $P_{Ltet0-1}$ control. Western blot analysis with α-TEV antibody from a 10% SDS-polyacrylamide gel. Samples were taken from a 1/100 diluted overnight culture of strain MC4100 pTH9 after 3 hours of growth in NZA medium containing 100 µg/ml spectinomycin. Expression was induced with 100 ng anhydrotetracycline (aTc).

4.1.1.1.2 Effect of the inducer anhydrotetracyclin on cell growth

Compared with tetracycline, anhydrotetracycline has an appromimately 100 fold lower antibiotic activity. To test whether a concentration of 100 ng anhydrotetracycline would have an effect on cell growth strain MC4100 was grown with and without the inducer (Fig. 4.29). The growth of the culture supplied with anhydrotetracycline was only slightly slower so that the effect of the inducer on cell growth was considered negligible.

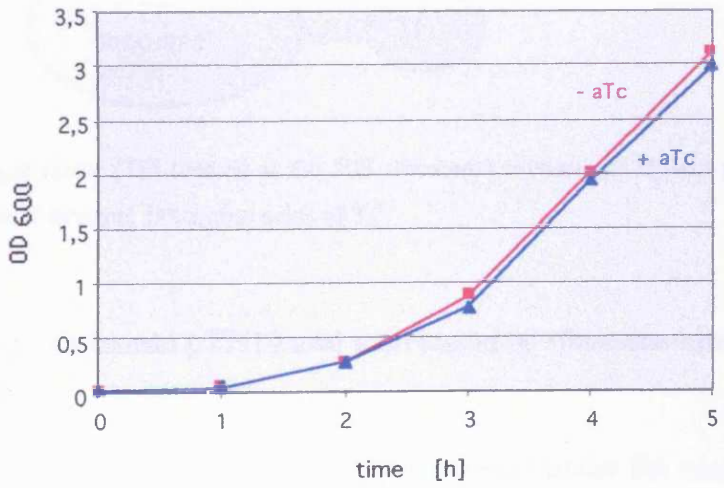


Fig. 4.29 Growth of strain MC4100. An overnight culture was diluted 1/100 in NZA medium with 100 ng anhydrotetracycline (aTc) or without the inducer.

4.1.3.3 Ribosome-attached TEV protease

Cleavage of a target protein by TEV protease was rendered more efficient by locating the protease in close proximity to the nascent polypeptide chain emerging from the ribosome. Trigger factor (TF) is a ribosome-associated chaperone that recognises unfolded polypeptide chains. TEV protease was located at the ribosome by fusing the 27 kDa NaI-Pro sequence of the protease to a short trigger factor fragment consisting of the first 144 amino acids (Fig. 4.30). This short fragment is sufficient for binding to the large ribosomal subunit near the exit for the nascent peptide chains. Subcloning of the *tig*144-TEV protease sequence resulting in plasmid pCC6 and confirmation of the correct localisation of the hybrid protein at the ribosome was done by Charlotte Conz (Conz, 2000)

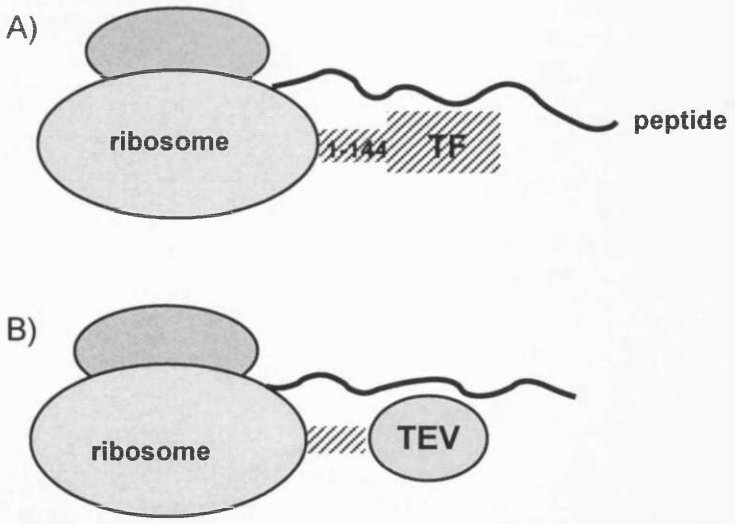


Fig. 4.30 A) Trigger factor (TF) located at the 50S ribosomal subunit. B) Hybrid protein consistent of TEV protease and the N-terminal 144 amino acids of TF.

4.1.3.3.1 Cloning of plasmid pTH10 and expression of ribosome-attached TEV protease

To bring the expression of the ribosomal TEV protease under the control of $P_{Ltet0-1}$ the *tig144*-TEV protease DNA fragment was amplified from plasmid pCC6 with oligonucleotide primers containing *Kpn I* or *Mlu I* restriction sites, respectively (Fig. 4.31). The PCR product of 1230 base pairs was cloned via these sites into a fragment of

pTH9. The resulting plasmid was named pTH10 and carried the *tig144*-TEV protease behind promoter $P_{Ltet0-1}$.

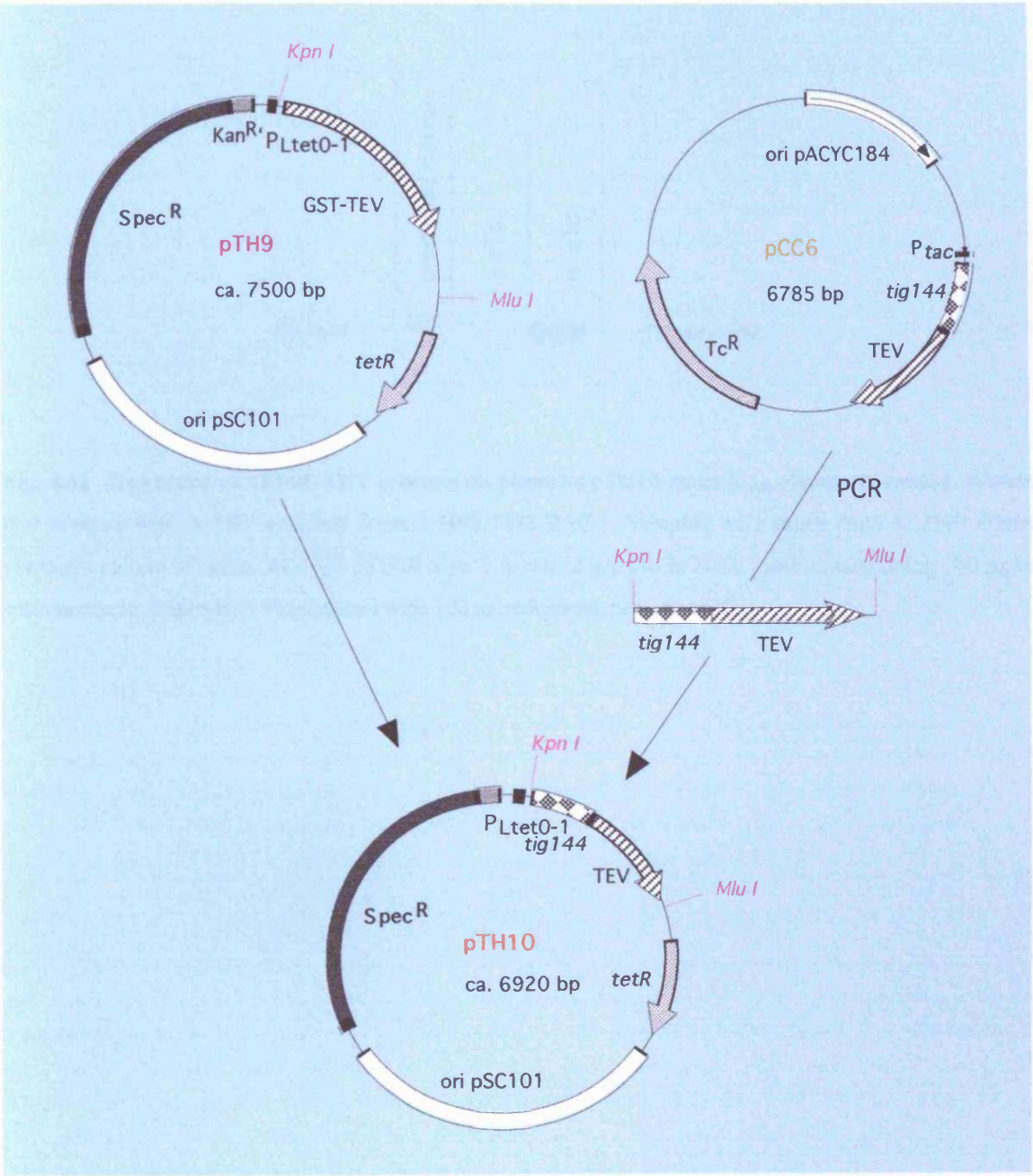


Fig. 4.31 Cloning of plasmid pTH10. The *tig*144 -TEV protease fragment from pCC6 was cloned into pTH9 after restriction digestion with the indicated enzymes. The sequences of the used oligonucleotide primers are listed in Table 3.8 in chapter 3.6.6.3

Expression of the TF144-TEV protease hybrid protein was tested in strain MC4100. On a Western blot the TF144-TEV band of approximately 46 kDa appeared upon induction with anhydrotetracycline, whereas no band was visible without the inducer (Fig. 4.32).

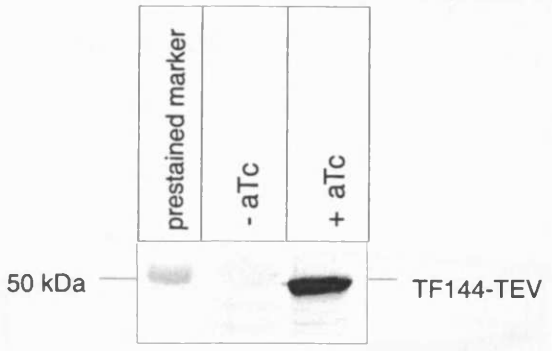


Fig. 4.32 Expression of TF144 -TEV protease on plasmids pTH10 under $P_{Ltet0-1}$ promoter control. Western blot analysis with α-TEV antibody from a 10% SDS-PAGE. Samples were taken from a 1/100 diluted overnight culture of strain MC4100 pTH10 after 3 hours of growth in NZA medium containing 100 µg/ml spectinomycin. Expression was induced with 100 ng anhydrotetracycline (aTc).

4.1.3.3.2 Effect of ribosome-attached TEV protease expression on cell growth

Strain MC4100 transformed with plasmid pCC6 (encoding the ribosomal TEV protease) grew as fast as MC4100 without the plasmid (Fig. 4.33). There was no difference in growth of the transformed strain with or without induction of TF144-TEV protease with 100 µM IPTG. This result indicated that the TEV protease had no severe effects for cell viability when it is located at the ribosome.

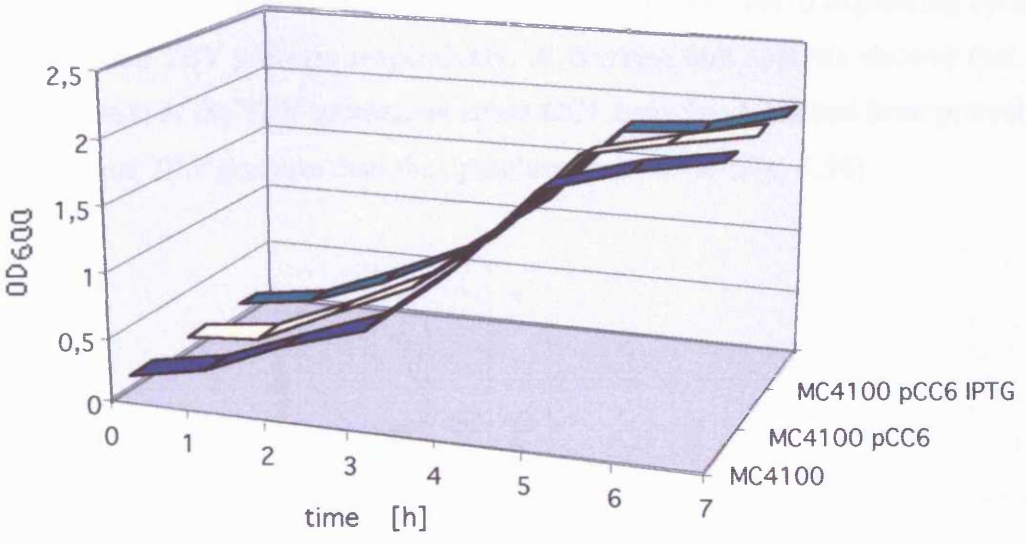


Fig. 4.33 Growth of strain MC4100 and MC4100 pCC6 (TF144-TEV protease) in M9 minimal medium containing 0.2% glucose, 1 µg/ml thiamine, 50 µg/ml 18 amino acid mix and 5 µg/ml tetracycline for the transformed strains. TEV protease expression was induced after 1 hours of growth with 100 µM IPTG.

4.1.3.4 *Proteolysis of SecA-PCS by cytoplasmic and ribosome-attached TEV protease*

The hypothesis that cleavage of nascent polypeptide chains by TEV protease is more effective than cleavage of the folded protein was tested in strains CC1 and CC2. These strains contain either *secA195* or *secA252* instead of wt *secA* and are regulated by the wt SecA promoter. Both strains also contain a deletion of the trigger factor gene (Δ*tig::kan*) to prevent competition for binding sites at the large ribosomal subunit between the wild-type trigger factor and the hybrid protein.

CC1 and CC2 were transformed with plasmids pTH9 and pTH10 expressing cytoplasmic and ribosomal TEV protease respectively. A Western blot analysis showed that 5 hours after induction of the TEV protease in strain CC1 more SecA252 had been proteolysed by the ribosomal TEV protease than the cytoplasmic protease (Fig. 4.34)

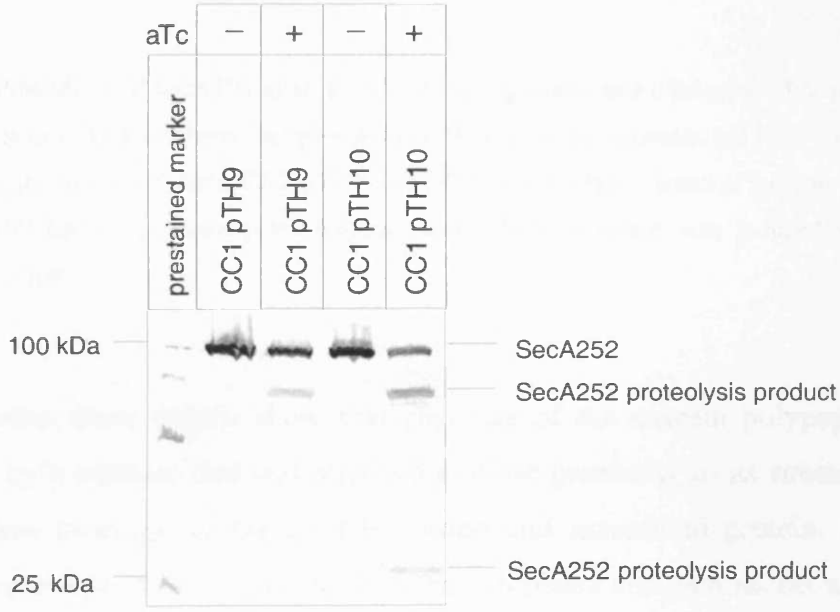


Fig. 4.34. Proteolysis of SecA252 in strain CC1 by cytoplasmic and ribosomal TEV protease. Western blot analysis with α-TEV antibody from a 10% SDS-polyacrylamide gel. Samples were taken from a 1/100 diluted overnight culture of strain CC1 pTH9 and CC1 pTH10 after 5 hours of growth in NZA medium containing 100 µg/ml spectinomycin. Expression of TEV protease was induced with 100 ng/ml anhydrotetracycline.

This result was even more obvious in strain CC2. After 5 hours of induction the amount of SecA195 was almost completely proteolysed by the ribosome-attached TEV protease (Fig. 4.35).

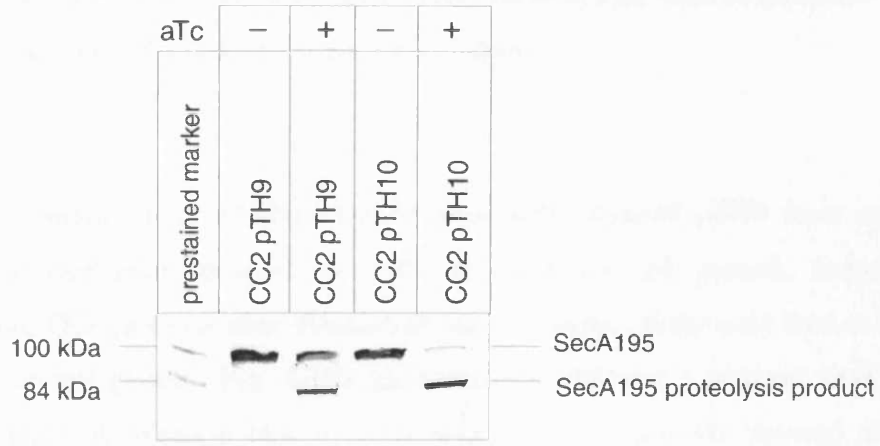


Fig. 4.35. Proteolysis of SecA195 in strain CC2 by cytoplasmic and ribosomal TEV protease. Western blot analysis with α-TEV antibody. Samples for a 10% SDS-polyacrylamide gel were taken from a 1/100 diluted overnight culture of strain CC2 pTH9 and CC2 pTH10 after 5 hours of growth in NZA medium containing 100 µg/ml spectinomycin. Expression of TEV protease was induced with 100 ng/ml anhydrotetracycline.

Taken together these results show that cleavage of the nascent polypeptide chain of SecA-PCS by a protease that was attached in close proximity to its substrate was more efficient than cleavage of the already folded and assembled protein. Soluble TEV protease first has to "find" its substrate in the cytoplasm and gain access to the cleavage site.

The reason why nascent SecA252 was not proteolysed as efficiently as SecA195 by the ribosome-attached TEV protease was not known.

4.1.3.5 Characterisation of potential SecA-inactivation strains

Strains TH25 pTH19, TH26 and TH27 were transformed with plasmids pTH9 and pTH10 expressing cytoplasmic or ribosomal TEV protease under $P_{Ltet0-1}$ regulation respectively. Growth of each strain was observed with and without induction of the TEV protease to screen for a possible inactivation strain.

SecA830

Cultures of strain TH25 pTHS19 transformed with plasmid pTH9 were supplied with IPTG to induce expression of SecA830 to facilitate cell growth. Induction of the cytoplasmic TEV protease after dilution of the overnight culture only lead to a very slight reduction in cell growth (Fig. 4.36). However, the difference between both curves was not significant. A Western blot analysis with α-SecA antibody showed proteolysis of SecA830 when TEV protease was induced with anhydrotetracycline (result not shown). The same result was obtained when ribosomal TEV protease was expressed on plasmid pTH10 (result not shown).

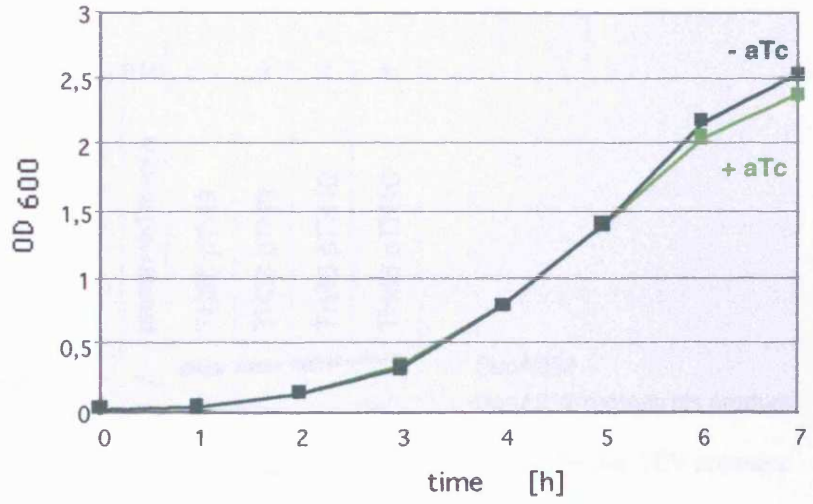


Fig. 4.36 Growth of strain TH25 pTHS19 pTH9. An overnight culture was grown in NZA medium with 25 µg/ml ampicillin, 5 µg/ml tetracycline and 100 µg/ml spectinomycin as well as 100 µM IPTG. The culture was diluted 1/100 into the same medium with 100 ng/ml aTc or without the inducer.

Obviously the proteolytic removal of the last 72 amino acids of SecA has no effect on cell growth. As this region contains the binding site for SecB this result leads to the assumption that either interaction of the two proteins is not essential for successful protein translocation or that a small amount of remaining uncleaved SecA830 is sufficient to maintain secretion of SecB-dependent proteins.

SecA252

Strain TH26 carrying SecA252 at the attachment site behind a non-regulatable *lac* promoter was transformed with pTH9. The trigger factor gene was disrupted by a kanamycin cassette via P1 transduction with a $P1_{CC4}$-lysate. The resulting strain was named TH46. This strain was then transformed with plasmid pTH10 expressing the TF144-TEV protease.

A Western blot analysis with α-SecA antibody and α-TEV protease antibody showed almost no difference in proteolysis efficiency between cytoplasmic and the ribosome-attached TEV protease after 5 hours of induction (Fig. 4.37). The expression of the respective protease hybrid protein induced with aTc is shown as well.

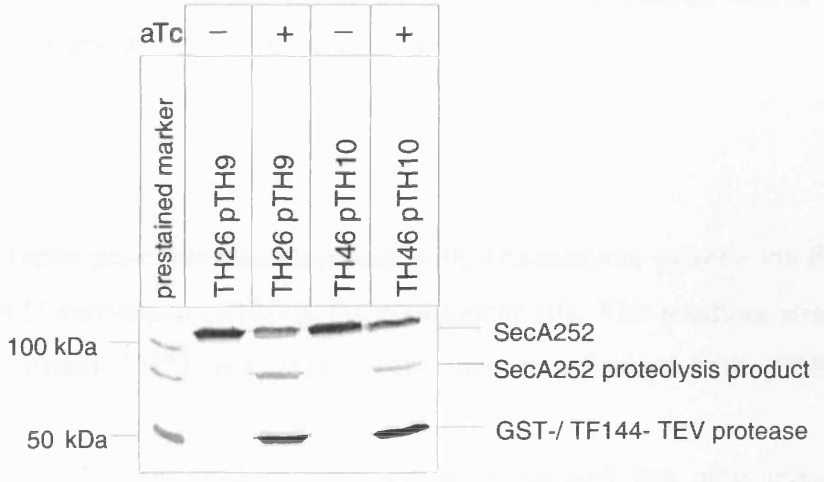


Fig. 4.37 Proteolysis of SecA252 in strain TH26 and TH46 (Δ*tig*::*kan*) by cytoplasmic and ribosomal TEV protease. Western blot analysis with α-SecA antibody and α-TEV antibody. Samples for a 10% SDS-PAGE were taken from a 1/100 diluted overnight culture of strain TH26 pTH9 and TH46 pTH10 after 5 hours of growth in NZA medium containing 100 µg/ml spectinomycin. Expression of TEV protease was induced with 100 ng/ml aTc after dilution.

Cell growth was not obviously slower when TEV protease was induced in either TH26 or TH46 (Fig. 4.38). Growth difference between TH26 and TH46 might be due to the disruption of trigger factor.

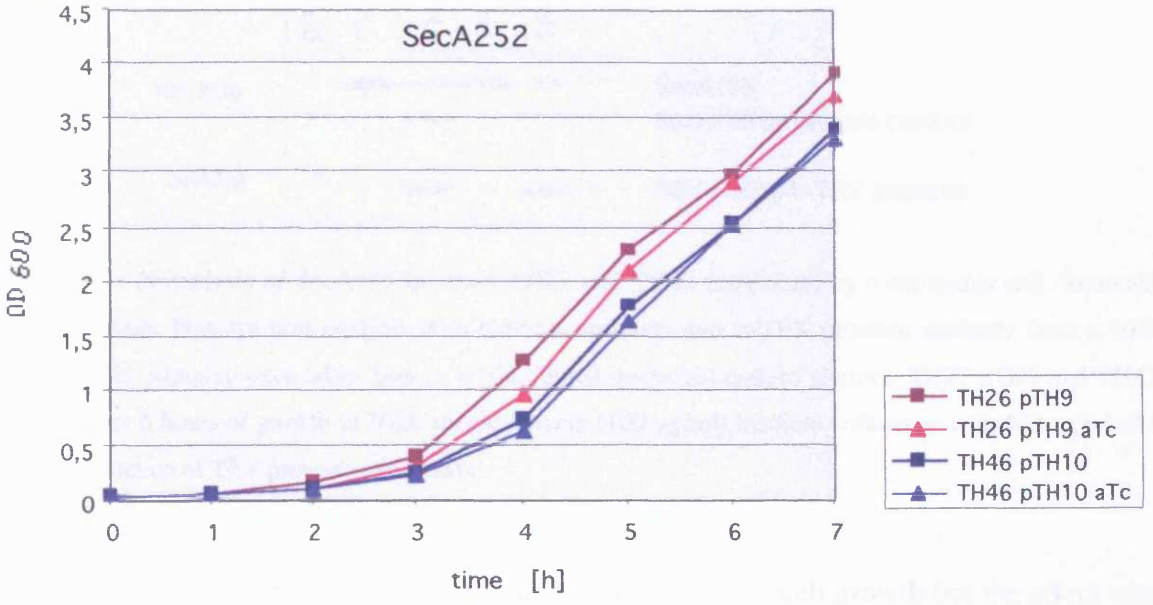


Fig. 4.38 Growth of strains TH26 pTH9 and TH46 pTH10. Overnight cultures grown in NZA medium with 100 µg/ml spectinomycin were diluted 1/100 into the same medium with or without inducer. Expression of TEV protease was induced with 100 ng/ml aTc.

SecA195

The trigger factor gene was also disrupted with a kanamycin cassette via P1 transduction in strain TH27 carrying *secA*195 at the attachment site. The resulting strain was named TH47. The strains TH27 and TH47 were then transformed with pTH9 and pTH10 respectively.

A Western blot analysis with α-SecA antibody showed that after induction of TEV protease for 5 hours SecA195 was much more efficiently cleaved by the ribosome-attached protease than by the cytoplasmic protease (Fig. 4.39).

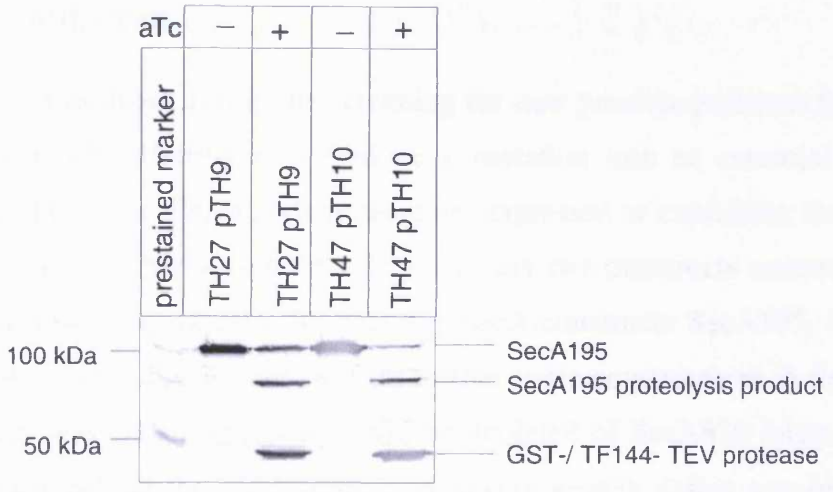


Fig. 4.39 Proteolysis of SecA195 in strain TH27 and TH47 (Δ*tig*::*kan*) by cytoplasmic and ribosomal TEV protease. Western blot analysis with α-SecA antibody and α-TEV protease antibody from a 10% SDS-PAGE. Samples were taken from a 1/100 diluted overnight culture of strain TH27 pTH9 and TH47 pTH10 after 5 hours of growth in NZA spectinomycin (100 µg/ml) medium without or with 100 ng/ml aTc for the induction of TEV protease expression.

Proteolysis of *secA*195 in both strains lead to a reduction in cell growth but the effect was much stronger when ribosomal TEV protease was expressed (Fig. 4.40). Growth was slowed down by a factor of about 3.

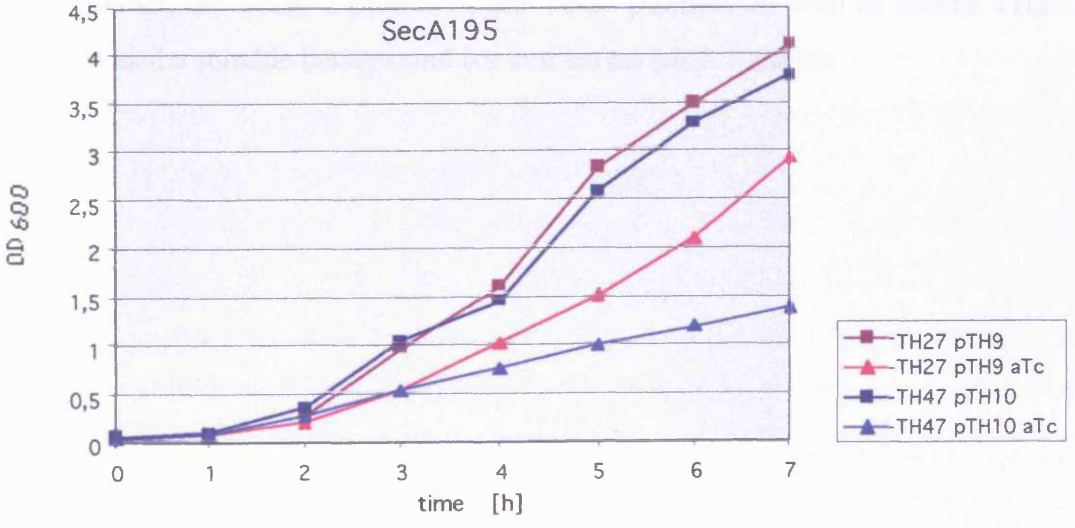


Fig. 4.40 Growth of strains TH27 pTH9 and TH47 pTH10. Overnight cultures grown in NZA spectinomycin medium were diluted 1/100 into the same medium with or without inducer (100 ng/ml aTc).

4.1.4 Conclusions

The problems that arose during the screening for new possible positions for the PCS in SecA showed the difficulty to introduce a mutation into an essential gene. Some constructs could not be cloned, others were not expressed or expression turned out to be unstable (Conz, 2000 and chapter 4.1.2.2). As only two constructs seemed to be stably expressed at low temperatures the existing SecA-constructs SecA195, SecA252 and SecA830 (Mondigler, 1997) were used for further strain constructions. A SecA-depletion strain (TH25) was constructed that could be depleted of SecA830 integrated at the λ attachment site behind the *lac* promoter. A severe growth defect was observed when expression of SecA830 was not induced. However, proteolysis of SecA830 had no negative effect on cell growth.

Although expression of SecA195 could not be regulated anymore, proteolysis of the SecA-PCS construct in strain TH47 by the TF144-TEV protease lead to a distinct reduction in cell growth. Proteolysis of SecA195 by TF144-TEV protease was more efficient than proteolysis by cytoplasmic TEV protease because the unfolded target protein was cleaved during translation at the ribosome. In addition, as TF144-TEV protease is also soluble in the cytoplasm SecA195 remains a target for proteolysis after translation is completed.

Taken together, the SecA-depletion strain TH25 pTHS19 as well as strains TH27 and TH47 provided a suitable background for studies on SecA function.

4.2 Effect of Ffh-, FtsY- and SecA-depletion or inactivation on secretion and membrane insertion

In *E. coli* the signal recognition particle, consiting of the Ffh protein and a 4.5S RNA, together with its receptor FtsY function in the targeting and insertion of membrane proteins. A role of these two components in protein secretion is thought to be rather minor. It was suggested that secretory proteins are targeted to the membrane and translocated via the SecB-SecA complex. A function of SecA in the insertion of non-secretory inner membrane proteins remains controversial. In an attempt to gain more information about the roles of Ffh, FtsY and SecA, biotinylation assays were performed with strains depleted of either of the components. Additional biotinylation assays were carried out using strains in which SecA could be effectively proteolysed by TEV protease.

4.2.1 Cloning of plasmids carrying PSPT hybrid proteins

Jander and coworkers developed a sensitive assay for detecting defects in the insertion of membrane proteins (Jander *et al.*, 1996). In this assay the biotin-accepting domain from the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase (PSBT) was fused to periplasmic domains of a membrane protein. When the correct secretion or insertion of the hybrid protein is prevented, the PSBT domain remains in the cytoplasm and is biotinylated by the cytoplasmic enzyme biotin ligase.

To study the dependency of some proteins on either Ffh, FtsY or SecA for secretion across or insertion into the cytoplasmic membrane the 76-amino acid PSBT fragment was fused to the C-terminal end of the secretory protein alkaline phosphatase, the periplasmic C-terminus of the small membrane protein FtsQ that possesses only one transmembrane domain and to two fragments of the membrane protein MalF. In the MalF-PSBT hybrid proteins the PSBT domain was fused behind the first N-terminal 66 amino acids or N-terminal 274 amino acids of MalF respectively. The residues C-terminal to the fusion joint were deleted. In the small MalF66 fragment the PSBT domain was fused to the short

second cytoplasmic domain between transmembrane domains 2 and 3. In the large MalF274 fragment the PSBT domain was fused to the C-terminus of the large second periplasmatic loop of about 180 amino acids (Fig. 4.41). When MalF274-PSBT is correctly inserted into the membrane the PSBT domain is localised in the periplasm. When insertion is impaired, this domain is retained in the cytoplasm or is translocated much slower and the enzyme biotin ligase has time to biotinylate the protein.

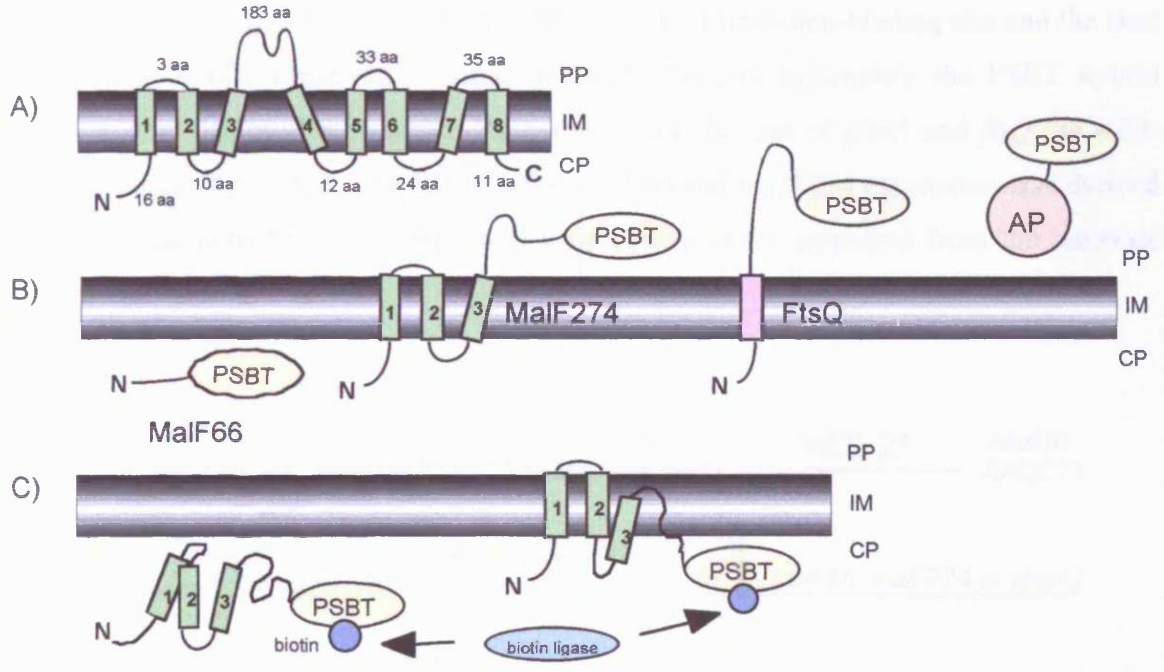


Fig. 4.41 A) Two dimensional topology model of MalF in the cytoplasmic membrane. B) Topologies of the hybrid proteins. The periplasmic PSBT domain cannot be biotinylated. C) Proposed localisations of the MalF274 hybrid protein when correct insertion of the protein is not possible or slowed down. The PSBT remains in the cytoplasm and is biotinylated by biotin ligase.

The performance of the biotinylation assays required the cloning of two series of plasmids carrying the PSBT hybrid proteins, as antibiotic resistance, type of promoter control and origin of replication had to be different from those carried on plasmids in the respective depletion strain.

4.2.1.1 *Plasmids expressing PSBT hybrid proteins under araBAD promoter control*

The first series of plasmids carrying either of the PSBT hybrid proteins were cloned as derivatives of plasmid pBAD18-Cm (Guzman *et al.*, 1995). This plasmid contains the P_{araBAD} promoter from the arabinose operon and its regulatory gene *araC*. The chloramphenicol resistance gene from pACYC184 is inserted into the *bla* gene. The multiple cloning site MCS 2* of pBAD18-Cm lacks a ribosome-binding site and the start codon (Fig. 4.42). Therefore, 5'oligonucleotide primers to amplify the PSBT hybrid proteins were designed with Shine-Dalgarno boxes. In case of *phoA* and *ftsQ* the wild-type SD boxes were used. The SD box for *malF*66 and *malF*274 expression was derived from plasmid pDHB32. The respective start codons were amplified from the template sequences.

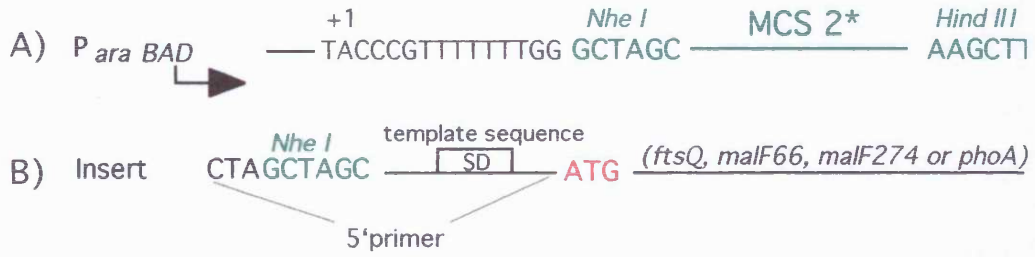


Fig. 4.42 A) Simplified region of MCS 2* on plasmid pBAD18-Cm and its derivatives. B) Illustration of the first part of the insert with 5'primer containing a *Nhe I* restriction site and a SD box. The 3'primer is not shown. Abbreviations: MCS= multiple cloning site, SD= Shine-Dalgarno box.

The cloning strategy for the pBAD18-Cm derived plasmids is shown in Figure 4.43. In a first step the *ftsQ*-PSBT sequence from plasmid pHP42 was amplified with primers 5*NheI*42 and 3*HindIII*42 and cloned into the MCS 2* of pBAD18-Cm via the *Nhe I* and *Hind III* sites. The resulting plasmid was named pTH20.

In a second step this plasmid was then used as a vector fragment for cloning plasmids pTH22, pTH23 and pTH24. The sequences of *malF*66, *malF*274 and *phoA* were amplified from plamids pDHB32 and pTH2 respectively with primers 5*malFNheI* and

3*malF*-S1*XbaI* or 3*malF*-L1*XbaI* as well as 5*phoANheI* and 3*phoAXbaI* (for primer sequences see Table 3.11 in chapter 3.6.6.3). The purified PCR products were inserted into the *Nhe I* and *Xba I* digested vector fragment of pTH20 to locate *malF*66, *malF*274 and *phoA* upstream of the PSBT sequence.

Plasmids pTH20, pTH22, pTH23 and pTH24 were control-digested using enzymes *Nhe I* and *Xba I* as well as *Hind III*. Correct fragment sizes were obtained on 0.9% agarose gels for all three plasmids (result not shown).

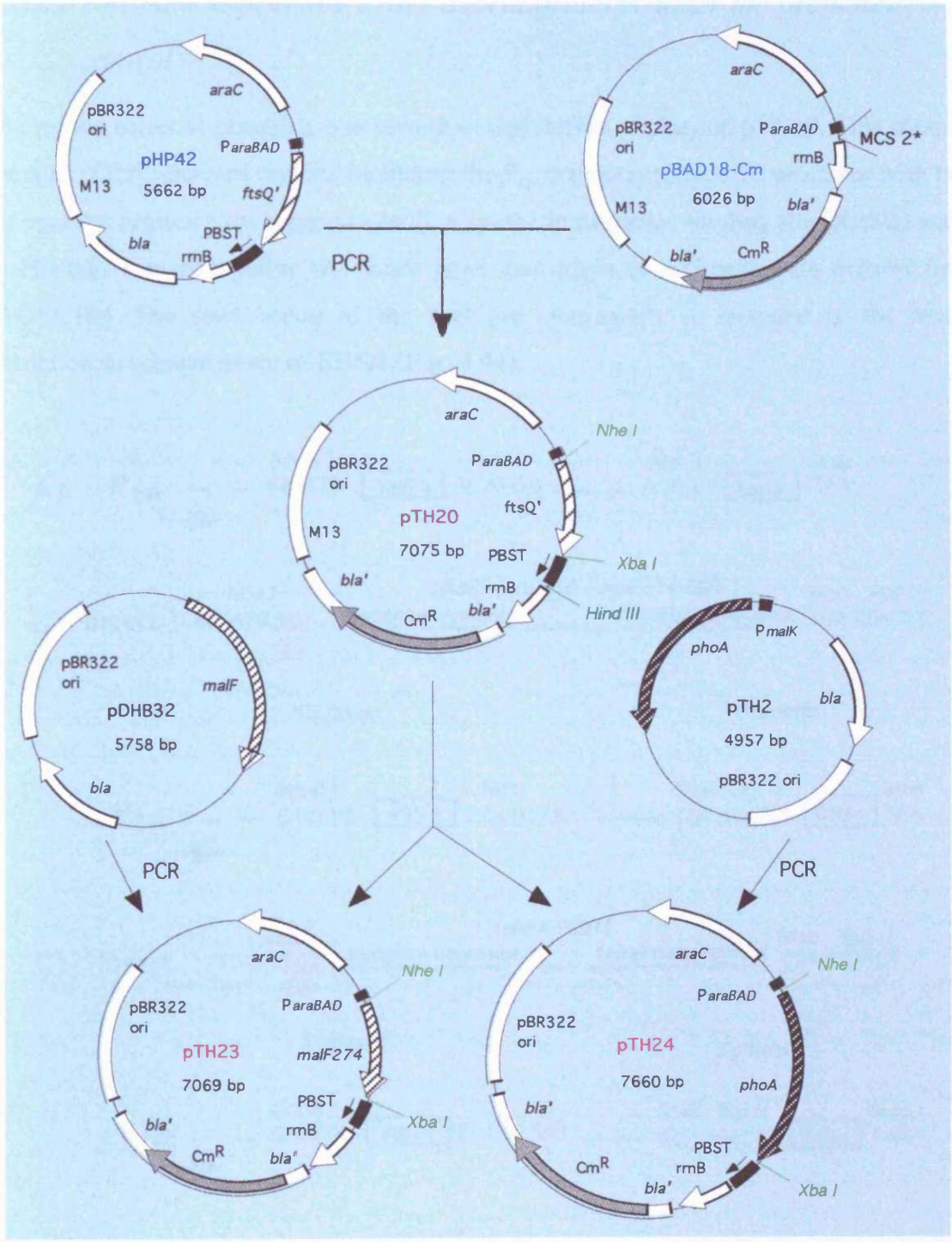


Fig. 4.43 Illustration of the cloning strategy for plasmids pTH20 (*ftsQ*-PSBT), pTH23 (*malF*274-PSBT) and pTH24 (*phoA*-PSBT). For details see text. Cloning of pTH22 is not shown but was performed similar to pTH23 construction. Restriction sites are indicated in green. The sequences of the oligonucleotide primers are listed in Table 3.11 in chapter 3.6.6.3

4.2.1.2 *Plasmids expressing PSBT hybrid proteins under tac promoter control*

The second series of plasmids was cloned as derivatives of plasmid pVC19. This plasmid contains pQE60-derived regions including the P_{tac} promoter (phage T5 promoter with two *lac* operator regions), the repressor *lacI*^{q}, a synthetic ribosome-binding site (RBSII) and a 6x His-tag. The tetracycline resistance gene and origin of replication are derived from pACYC184. The start codon of the multiple cloning-site is included in the *Nco I* restriction site downstream of RBSII (Fig. 4.44).

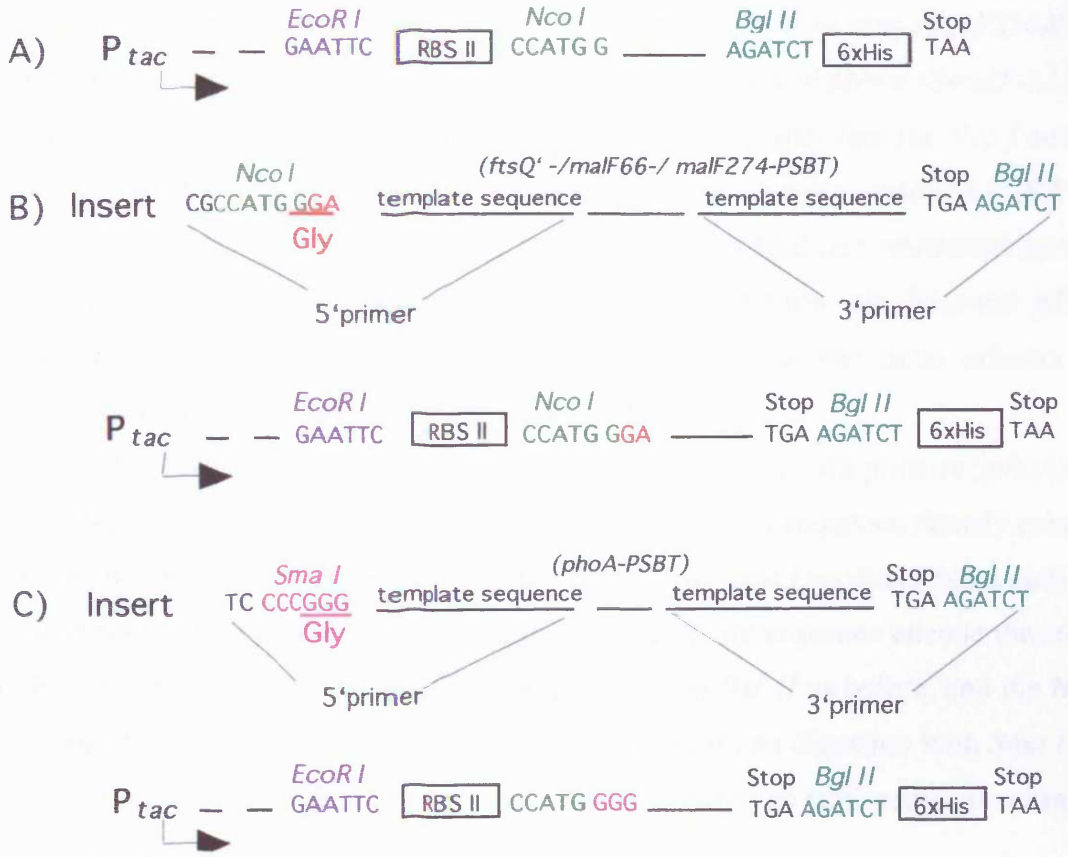


Fig. 4.44 A) Simplified promoter and multiple cloning site region in pVC19. B) Illustration of the *ftsQ*-PSBT and *malF*66-, *malF*274-PSBT inserts with 5'primer and 3'primer as well as the promoter region after insertion via the indicated sites (green). C) Illustration of the *phoA*-PSBT insert with 5'primer and 3'primer. The promoter region is shown after the insertion of the PCR fragment into the Klenow filled *Nco I* site. Start codon in *Nco I* site is marked bold. Abbreviations: RBSII = ribosome-binding site.

The cloning strategy for the PSBT hybrid proteins behind P_{tac} is shown in Figure 4.45. The *ftsQ*-PSBT fragment was amplified from plasmid pHP42 with primers 5*NcoI*42-gly and 3*BglII*42 and cloned into the *Nco I* and *Bgl II* sites of pVC19 resulting in plasmid pTH25. As illustrated in Figure 4.44 the 5'primer was designed with two additional nucleotides between the *Nco I* site and the beginning of the template sequence. This was necessary to shift the sequence into the correct reading frame. Together with the last guanine of the *Nco I* site these nucleotides encode the smallest amino acid, glycine, to keep the alteration in the newly synthesised polypeptide as small as possible. The 6x His-tag was not relevant for the purposes here. Therefore, the 3' primers contained a stop codon upstream of the *Bgl II* site.

Plasmid pTH23 served as a template for the amplification of the *malF*274-PSBT fragment using primers with *Nco I* and *Bgl II* sites as described above (5*malF*-L1*NcoI* and 3*BglII*42). The insert fragment was then cloned via these sites into the *Nco I* and *Bgl II* digested vector fragment of pTH25. The new plasmid carrying *malF*274-PSBT was named pTH28. The PSBT sequence had to be PCR amplified and reinserted into the vector fragment as pVC19 already carries a *Xba I* restriction site. Plasmid pTH27 carrying *malF*66-PSPT was cloned likewise to pTH28 with the same primers and restriction sites but using pTH22 as template (not shown).

The *phoA*-PSBT sequence was amplified from plasmid pTH24 with primers 5*phoASmaI* and 3*BglII*42. A different 5'primer had to be used as the *phoA* sequence already contains a *Nco I* restriction site (Fig. 4 44). The restriction enzyme *Sma I* produces blunt ends and the remaining three nucleotides connected with the template sequence encode the amino acid glycine. Vector pTH25 was digested with *Nco I* and *Bgl II* as before, and the *Nco I* site was filled by the Klenow fragment. After purification and digestion with *Sma I* and *Bgl II* the *phoA*-PSBT PCR fragment was cloned into the pTH25 vector resulting in plasmid pTH29.

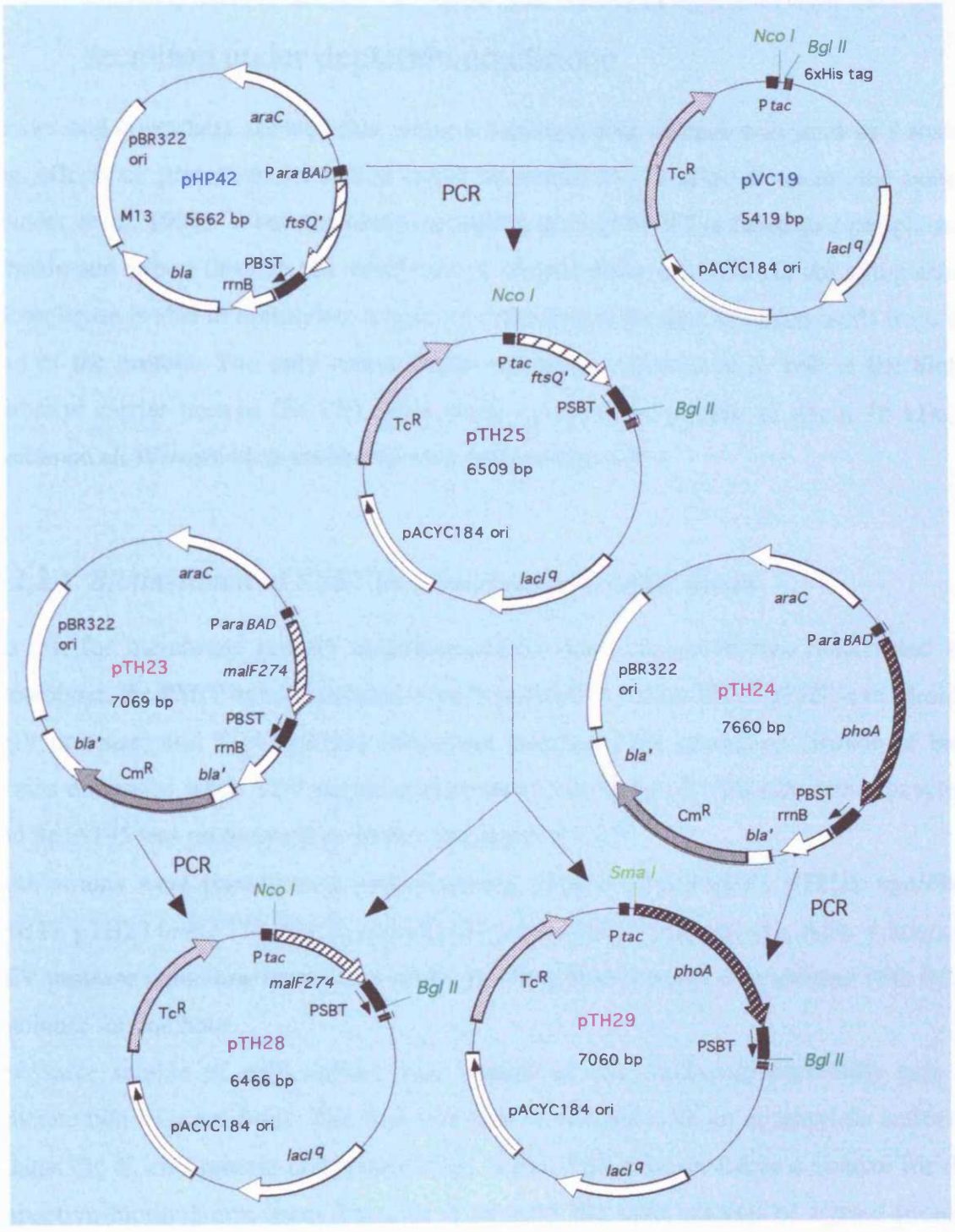


Fig. 4.45 Illustration of the cloning strategy for plasmids pTH25 (*ftsQ*-PSBT), pTH28 (*malF*274-PSBT) and pTH29 (*phoA*-PSBT). For details see text. Restriction sites are indicated in green. For the sequences of the oligonucleotide primers see Table 3.11 in chapter 3.6.6.3

4.2.2 Biotinylation-assays to indicate membrane insertion or secretion under depletion conditions

Jander and coworkers showed that when a biotinylatable domain was used as a fusion tag, effects on protein translocation could be sensitively detected in an *in vivo* system (Jander *et al.*, 1996). When the biotin-accepting domain PSBT is fused to a periplasmic domain and export through the membrane is slowed down or inhibited, the cytoplasmic biotin ligase is able to biotinylate a lysine residue that is located 35 amino acids from the end of the protein. The only native biotin containing protein in *E. coli* is the biotin carboxyl carrier protein (BCCP). This small cytoplasmic protein of about 16 kDa is visible on all Western blots incubated with streptavidin-AP.

4.2.2.1 Biotinylation of PSBT in a SecA- inactivation strain

To test for membrane protein insertion defects when SecA195 was inactivated via proteolysis, the PSBT hybrid proteins were expressed in strains TH27 pTH9 (cytoplasmic TEV protease) and TH47 pTH10 (ribosome-attached TEV protease). Growth of both strains decreased when TEV protease expression was induced with anhydrotetracycline and SecA195 was proteolysed as shown in chapter 4.1.3.5.

Both strains were transformed with plasmids pTH20 (*ftsQ*-PSBT), pTH22 (*malF*66-PSBT), pTH23 (*malF*274-PSBT) and pTH24 (*phoA*-PSBT) respectively. After 4 hours of TEV protease induction, expression of the PSBT hybrid proteins was induced with 0.2% arabinose for one hour.

The same sample of each culture was loaded on two SDS-polyacrylamide gels to generate two Western blots. The first blot was developed with an appropriate antibody against the *E. coli* protein component (Fig. 4.46). This blot served as a control for the respective biotinylation assay because it showed the total amount of hybrid protein expressed under experimental conditions. The second Western blot was developed with streptavidin-AP for the detection of biotinylated hybrid protein. However, expression of FtsQ-PSBT could not be tested, as no α-FtsQ antibody was available. Also, expression of Mal66-PSBT could not be shown, as the fragment is too short to be recognised by the α-

MalF antibody. Nevertheless, in strain TH27 pTH10 AP-PSBT and MalF274-PSBT were inducible with arabinose under test conditions (+ aTc, SecA195 inactivation by TEV protease) and control conditions (- aTc). A small amount of protein was synthesised without arabinose induction. As reported elsewhere, the *araBAD* promoter was not always tightly regulated (Seigle and Hu, 1998). Expression of the two proteins in strain TH27 pTH9 showed the same pattern on a Western blot (result not shown).

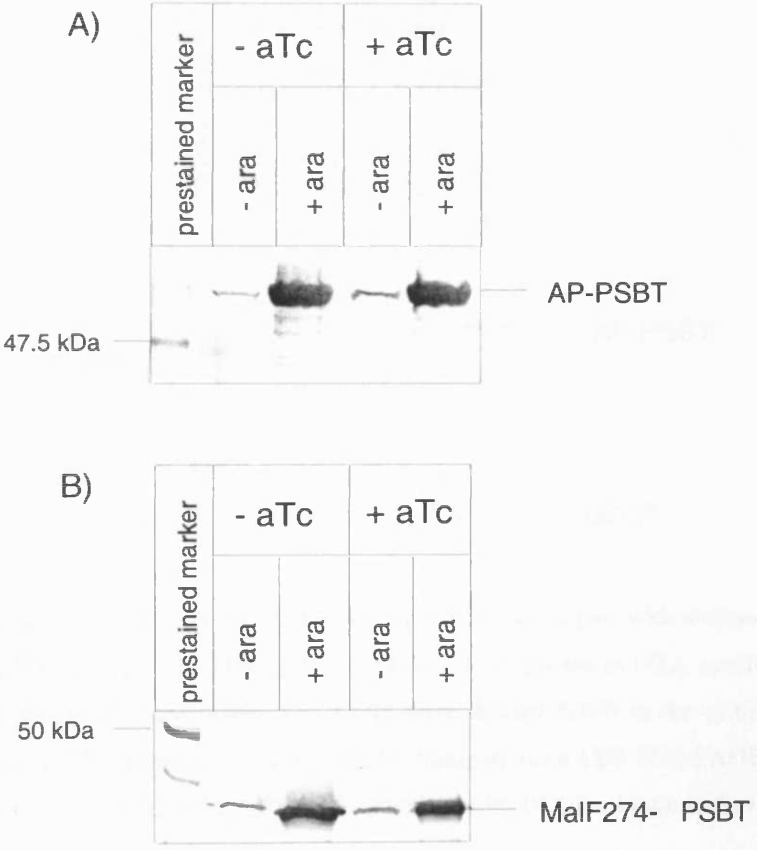


Fig. 4.46 Expression of AP-PSBT and MalF274-PSBT in TH47 pTH10 pTH23 and pTH24. A) Western blot from a 10% SDS-PAGE with α-AP antibody. B) Western blot with α-MalF antibody from a 10% SDS-PAGE. An overnight culture of TH47 pTH10 pTH24 or pTH23 was grown in NZA medium with 100 µg/ml spectinomycin and 30 µg/ml chloramphenicol. Cells were diluted 1/100 in the same medium with 100 ng/ml aTc (induction of *tig*144-TEV protease) or without aTc. After 4 hours of growth samples were divided and expression of the PSBT-hybrid protein was induced with 0.2% arabinose in one half of the samples for 1 hour.

The AP-PSBT protein was biotinylated in strain TH47 pTH10 when SecA195 was inactivated by TF144-TEV protease. This result was more obvious when AP-PSBT was not overexpressed by induction with arabinose (Fig. 4.47) as the high amount of protein probably overloaded the translocation system (result not shown). Under SecA195 inactivation conditions in strain TH27 pTH9 the amount of biotinylated protein was smaller. This result showed again that proteolysis of SecA195 is more efficient by the TF144- TEV protease.

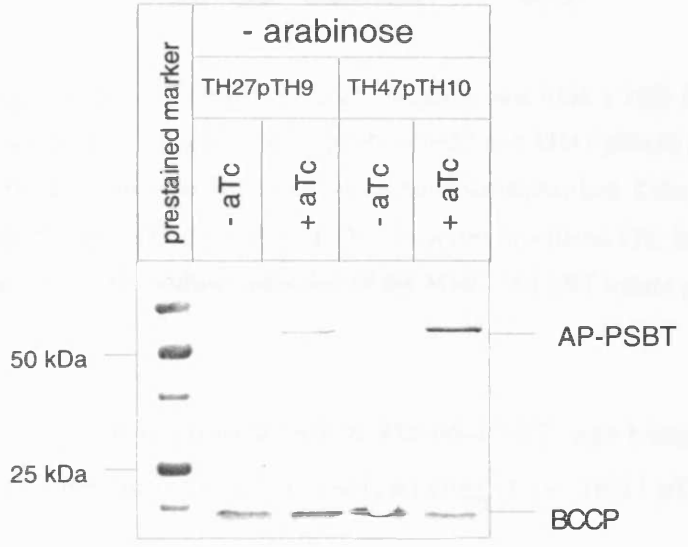


Fig. 4.47 Detection of biotinylated AP-PSBT. Western blot developed withstreptavidin-AP. Overnight cultures of TH27 pTH9 pTH24 and TH47 pTH10 pTH24 were grown in NZA medium with 100 µg/ml spectinomycin and 30 µg/ml chloramphenicol. Cells were diluted 1/100 in the same medium with 100 ng/ml aTc (induction of TEV protease) or without aTc. Samples for a 12% SDS-PAGE were taken after 5 hours of growth without induction of the AP-PSBT hybrid protein. BCCP = biotin carboxyl carrier protein.

Biotinylation of MalF274-PSBT showed a similar pattern as biotinylation of AP-PSBT. When SecA195 was inactivated by TEV protease the hybrid protein was biotinylated (Fig. 4.48). This effect was stronger in strain TH47 pTH10 expressing the ribosome-attached TEV protease. Again, MalF274-PSBT was not induced with arabinose, as the amount of hybrid protein synthesised without inducer was enough to indicate the insertion defect by biotinylation.

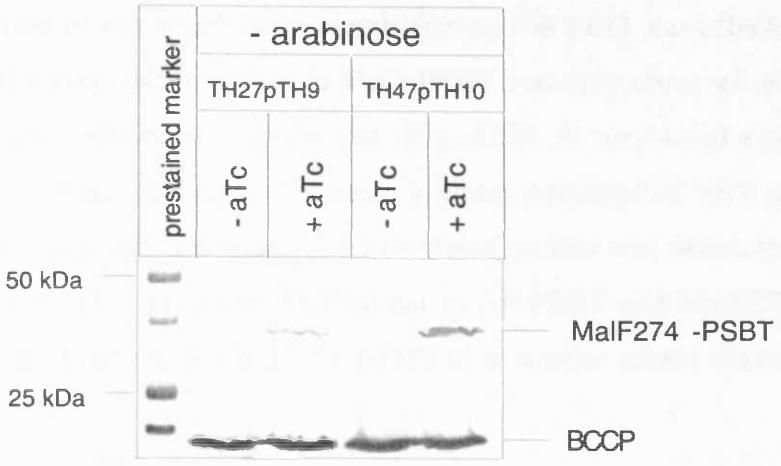


Fig. 4.48 Detection of biotinylated MalF274-PSBT. Western blot from a 12% SDS-PAGE developed with streptavidin-AP. Overnight cultures of TH27 pTH9 pTH23 and TH47 pTH10 pTH23 were grown in NZA medium with 100 µg/ml spectinomycin and 30 µg/ml chloramphenicol. Cells were diluted 1/100 in the same medium with 100 ng/ml aTc (induction of TEV protease) or without aTc. Samples for SDS-PAGE were taken after 5 hours of growth without induction of the MalF274-PSBT hybrid protein. BCCP = biotin carboxyl carrier protein.

As expected, the cytoplasmic control-fusion Mal66-PSBT was biotinylated heavily in both strains independently of SecA195 proteolysis (Fig. 4.49, TH27 pTH9 not shown).

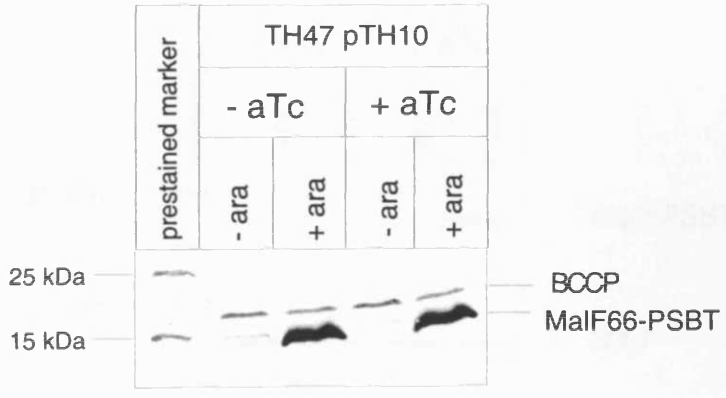


Fig. 4.49 Detection of biotinylated MalF66-PSBT. Western blot developed withstreptavidin-AP. Overnight culture of TH47 pTH10 pTH22 was diluted 1/100 in NZA spectinomycin (100 µg/ml) chloramphenicol (30 µg/ml) medium with 100 ng/ml aTc (induction of TEV protease) or without aTc. After 4 hours of growth, samples were divided and expression of MalF66-PSBT was induced with 0.2% arabinose in one half of the samples for 1 hour. BCCP = biotin carboxyl carrier protein.

Even the insertion of the small inner membrane protein FtsQ was affected by SecA195 inactivation. However, biotinylation of FtsQ-PSBT was only observed when expression of the protein was induced with arabinose (Fig. 4.50). A very small amount of protein was biotinylated also in the control culture without induction of TEV protease (-aTc). However, a distinctly higher amount of biotinylated protein was detected when SecA195 was inactivated by TEV protease. In contrast to AP-PSBT and MalF274-PSBT, FtsQ-PSBT was biotinylated in strain TH27 pTH9 to a similar extent than in strain TH47 pTH10.

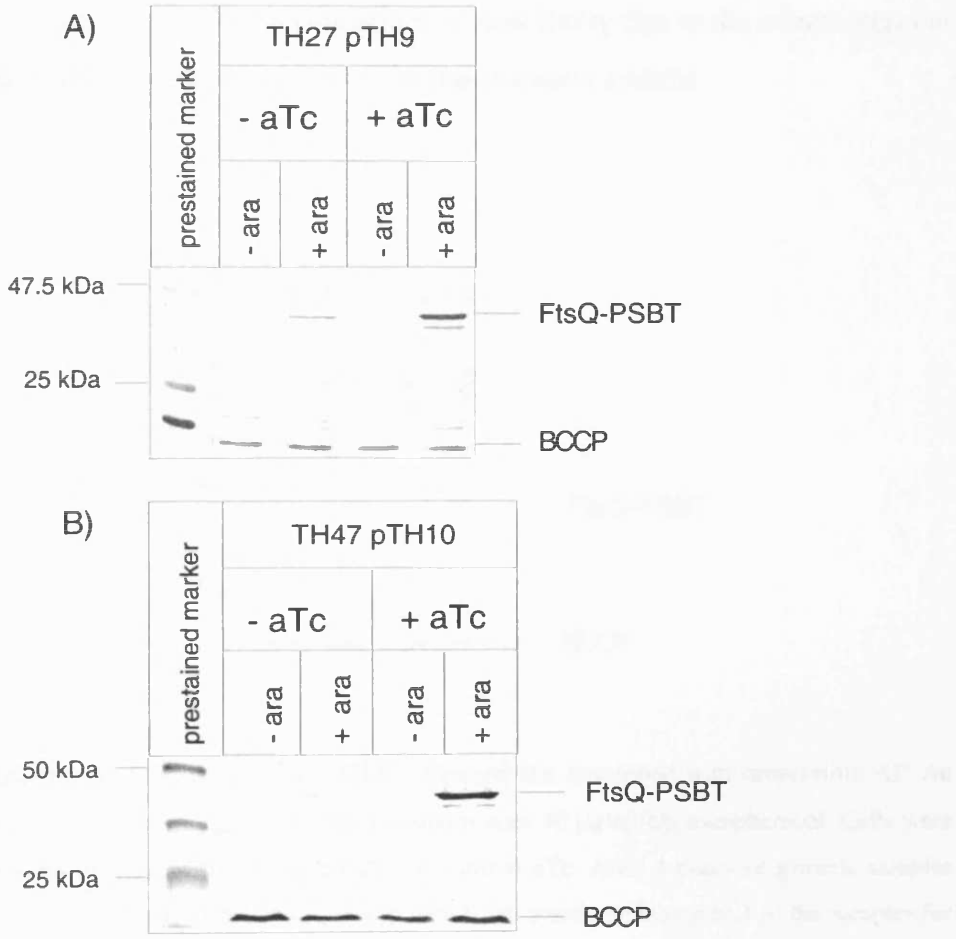


Fig. 4.50 Biotinylation of FtsQ-PSBT A) in TH27 pTH9 B) in TH47 pTH10. Western blot developed with streptavidin-AP. An overnight culture of the strains with pTH20 was grown in NZA medium with 100 µg/ml spectinomycin and 30 µg/ml chloramphenicol. Cells were diluted 1/100 in the same medium with 100 ng/ml aTc (induction of GST-TEV protease or TF144-TEV protease) or without aTc. After 4 hours of growth, samples were divided and expression of FtsQ-PSBT was induced with 0.2% arabinose in one half of the samples for 1 hour. Samples were loaded on a 12% SDS-PAGE.

The following control was performed to test whether the proteolysis of SecA195 and not the addition of the inducer aTc was responsible for the observed biotinylation of the PSBT hybrid protein. Strain TH47 was transformed with plasmid pTH20 (encoding *ftsQ*-PSBT). The biotinylation experiment showed that the appearance of a biotinylated protein band was independent of the addition of aTc when no TEV protease was present in the cells (Fig. 4.51). Therefore, inactivation of SecA195 via proteolysis is responsible for an impairment of FtsQ-PSBT translocation resulting in the biotinylation of the PSBT domain in the cytoplasm. When FtsQ-PSBT was induced with 0.2% arabinose a thin band was visible on the Western blot. This effect is most likely due to the overexpression of the protein and therefore oversaturation of the translocation system.

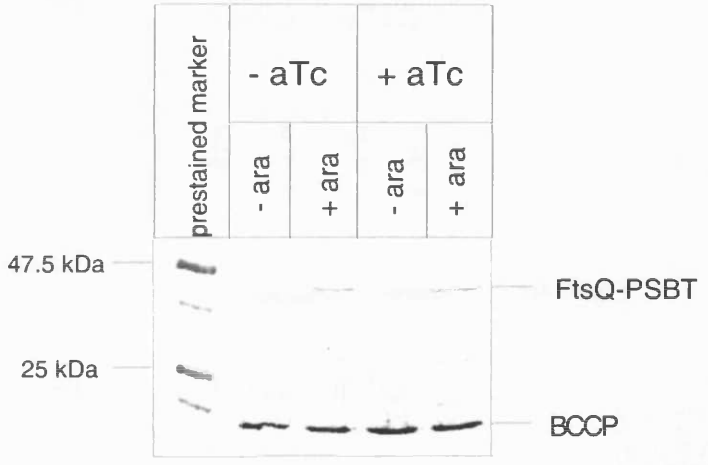


Fig. 4.51 Biotinylation of FtsQ-PSBT in strain TH47. Western blot developed with streptavidin-AP. An overnight culture of TH47 pTH20 was grown in NZA medium with 30 µg/ml chloramphenicol. Cells were diluted 1/100 in the same medium with 100 ng/ml aTc or without aTc. After 4 hours of growth, samples were divided and expression of FtsQ-PSBT was induced with 0.2% arabinose in one half of the samples for 1 hour. Samples were loaded on a 12% SDS-PAGE prior to blotting. BCCP = biotin carboxyl carrier protein

4.2.2.2 Biotinylation of PSBT in a SecA-depletion strain

The SecA-dependency of secretion of AP and membrane insertion of FtsQ and MalF274 was also examined in a SecA-depletion strain. SecA830 was depleted in strain TH25 pTHS19 carrying the SecA-PCS construct under P_{tac} control inserted at the λ attachment site and the *lacI*^{q} gene on plasmid pTHS19. Depletion (growth without IPTG supplementation) is shown in chapter 4.1.1.19. As growth without IPTG ceased after about 5 hours, SecA830 was depleted for 5.5 hours prior to the induction of the hybrid proteins with 0.2% arabinose in the transformed strain. Strain TH25 pTHS19 transformed with pTH23 and pTH24 showed regulatable expression of MalF274-PSBT and AP-PSBT respectively under depletion and non-depletion conditions (Fig. 4.52).

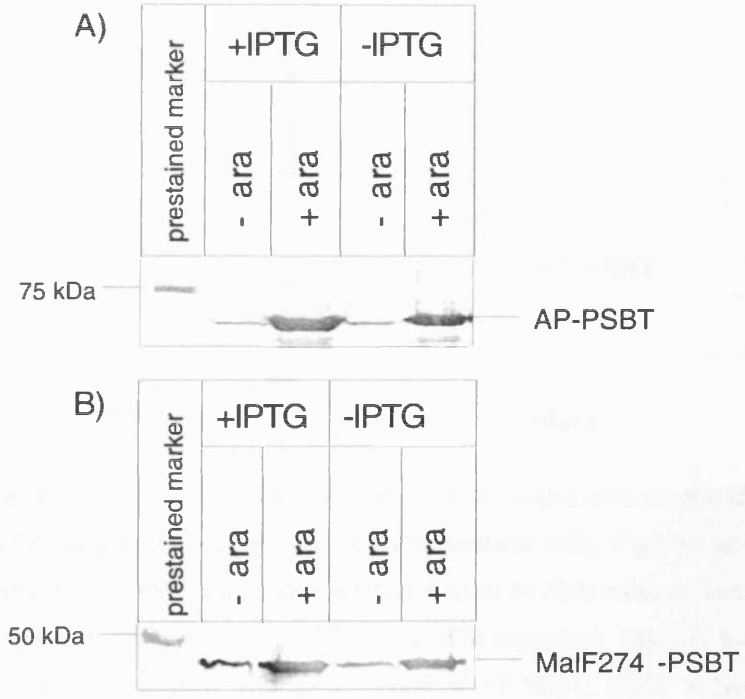


Fig. 4.52 Expression of AP-PSBT and MalF274-PSBT in TH25 pTHS19 pTH23 and pTH24. A) Western blot with α-AP antibody. B) Western blot with with α-MalF antibody. An overnight culture of TH25 pTHS19 pTH23 or pTH24 was grown in NZA tetracycline (5 μg/ml) chloramphenicol (30 μg/ml) medium and 100 μM IPTG. Cells were washed 3 times in NZA medium and diluted 1/100 in the same medium with or without IPTG (100 μM). After 5.5 hours of growth samples were divided and expression of AP-PSBT was induced with 0.2% arabinose in one half of the samples. Samples for a 10% SDS-PAGE were taken 1 hour after induction.

Without arabinose in the medium a small amount of protein was synthesised, showing that P_{araBAD} is not absolutely tight. The amount of protein increased upon induction with 0.2% arabinose.

As observed in the SecA-inactivation strains full induction of the AP-PSBT protein with 0.2% arabinose caused very intense biotinylated bands under depletion as well as non-depletion conditions (result not shown). As arabinose is actively transported into the cell a fine-tuned induction for a more subtle effect is not possible. However, the effect of SecA830 depletion on AP export was clearly detectable even without arabinose supplementation (Fig. 4.53) because a small amount of the AP-PSBT is expressed without induction (Fig. 4.52).

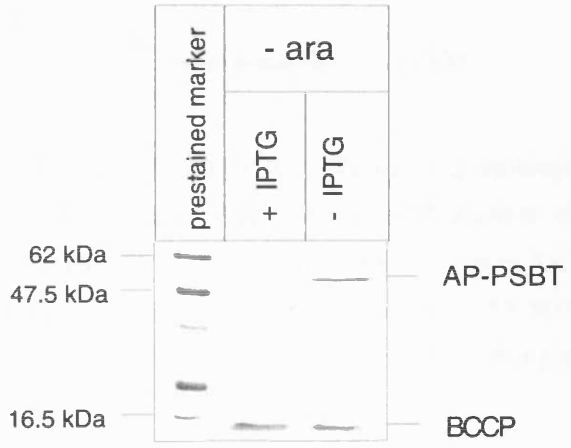


Fig. 4.53 Detection of biotinylated AP-PSBT. Western blot developed with streptavidin-AP. An overnight culture of TH25 pTHS19 pTH24 was grown in NZA medium with 5 µg/ml tetracycline, 30 µg/ml chloramphenicol and 100 µM IPTG. Cells were washed 3 times in NZA medium and diluted 1/100 in the same medium with 100 µM IPTG or without IPTG (SecA830 depletion). Samples for a 12% SDS-PAGE were taken after 6.5 hours of growth without induction of AP-PSBT. BCCP = biotin carboxyl carrier protein.

Not only the secretory protein AP was affected by SecA830-depletion but also MalF274-PSBT was biotinylated when IPTG was not supplemented in the medium (Fig. 4.54). As with AP-PSBT, the effect was clearer when MalF274 was not overexpressed by arabinose-induction.

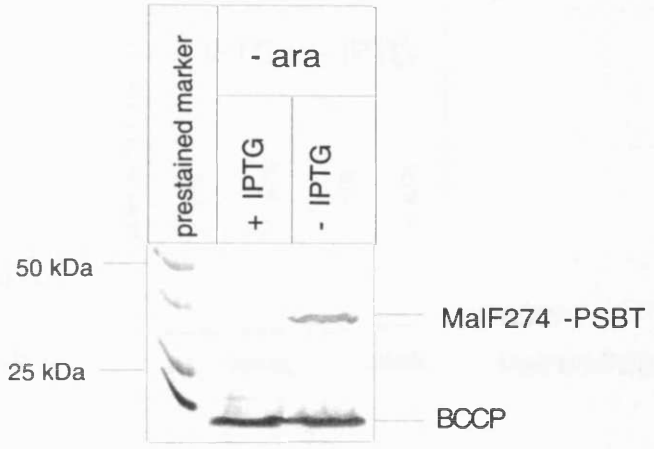


Fig. 4.54 Detection of biotinylated MalF274-PSBT. Western blot developed with streptavidin-AP. An overnight culture of TH25 pTHS19 pTH23 was grown in NZA medium with 5 µg/ml tetracycline, 30 µg/ml chloramphenicol and 100 µM IPTG. Cells were washed 3 times in NZA medium and diluted 1/100 in the same medium with or without IPTG (100 µM). Samples for a 12% SDS-PAGE were taken after 6.5 hours of growth without induction of MalF274-PSBT. BCCP = biotin carboxyl carrier protein.

The cytoplasmic control hybrid protein MalF66-PSBT (15 kDa) was biotinylated when SecA830 was depleted (- IPTG) as well as under "wild-type"-conditions when IPTG was supplemented (Fig. 4.55). A small amount of biotinylated protein was detectable without induction of MalF66-PSBT due to the leakiness of the *araBAD* promoter.

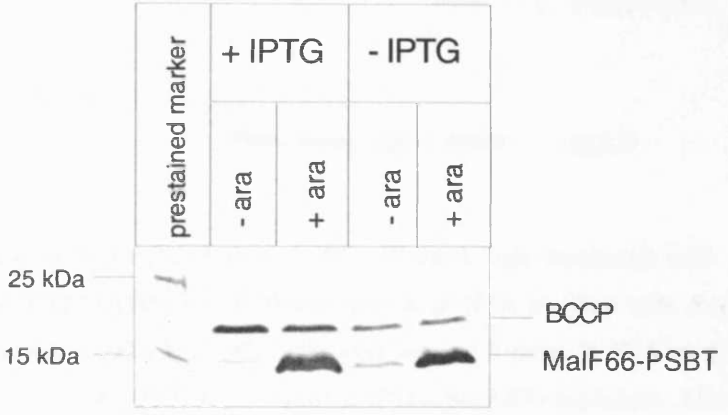


Fig. 4.55 Biotinylation of MalF66-PSBT. Western blot developed with streptavidin-AP. An overnight culture of TH25 pTHS19 pTH22 was grown in NZA medium with 5 µg/ml tetracycline, 30 µg/ml chloramphenicol and 100 µM IPTG. Cells were washed 3 times in NZA medium and diluted 1/100 in the same medium with 100 µM IPTG or without IPTG (SecA830-depletion). After 5.5 hours of growth samples were divided and expression of MalF66-PSBT was induced with 0.2% arabinose in one half of the samples. Samples for a 12% SDS-PAGE were taken after 1 hour of induction. BCCP = biotin carboxyl carrier protein.

As observed in the SecA-inactivation strain, also the insertion of FtsQ was affected by SecA830 depletion. Biotinylation of FtsQ-PSBT was only observed when expression of the protein was induced with arabinose (Fig. 4.56). As in contrast to AP-PSBT and MalF274-PSBT a higher amount of hybrid protein was necessary for the detection of the biotinylation effect, the dependency on SecA for FtsQ-insertion might not be as strong. Another explanation might be that FtsQ is a much better substrate for SecA so that only higher amounts of the protein resulted in detectable defect of its insertion.

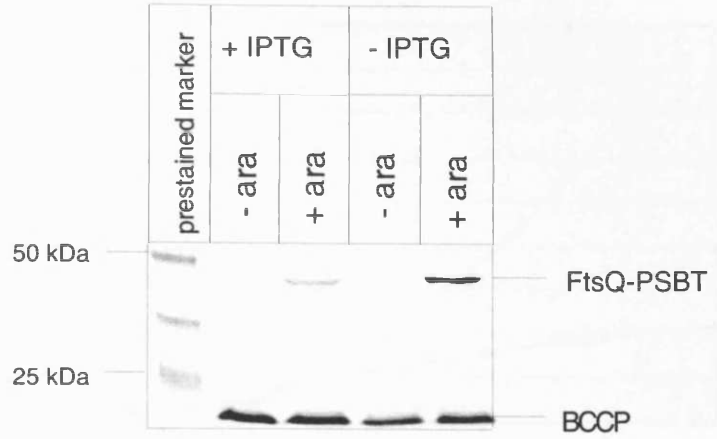


Fig. 4.56 Detection of biotinylated FtsQ-PSBT. Western blot incubated with streptavidin-AP. An overnight culture of TH25 pTHS19 pTH20 was grown in NZA medium with 5 µg/ml tetracycline, 30 µg/ml chloramphenicol and 100 µM IPTG. Cells were washed 3 times in NZA medium and diluted 1/100 in the same medium with 100 µM IPTG or without IPTG (SecA830-depletion). After 5.5 hours of growth samples were divided and expression of FtsQ-PSBT was induced with 0.2% arabinose in one half of the samples. Samples for a 12% SDS-PAGE were taken after 1 hour of induction. BCCP = biotin carboxyl carrier protein.

4.2.2.3 Conditions of Ffh-depletion

The effects on biotinylation of AP-PSBT, MalF274-PSBT and FtsQ-PSBT were also studied in the Ffh-depletion strain WAM113 (Phillips and Silhavy, 1992). This strain contains a chromosomal knockout mutation in *ffh* (*ffh*::*kan*) and a chromosomal copy of the *ffh* gene under the control of the *araBAD* promoter. Growth of WAM113 ceased after 3 hours in the arabinose free medium (Fig. 4.57) and by this time Ffh was not detectable on a Western blot anymore (Fig. 4.58).

Therefore, after 3 hours of growth of a transformed WAM113 strain, expression of the PSBT-hybrid proteins was induced. After one hour of induction samples were taken for Western blot analysis and detection of biotinylated hybrid proteins via streptavidin-AP and BCIP/NBT development.

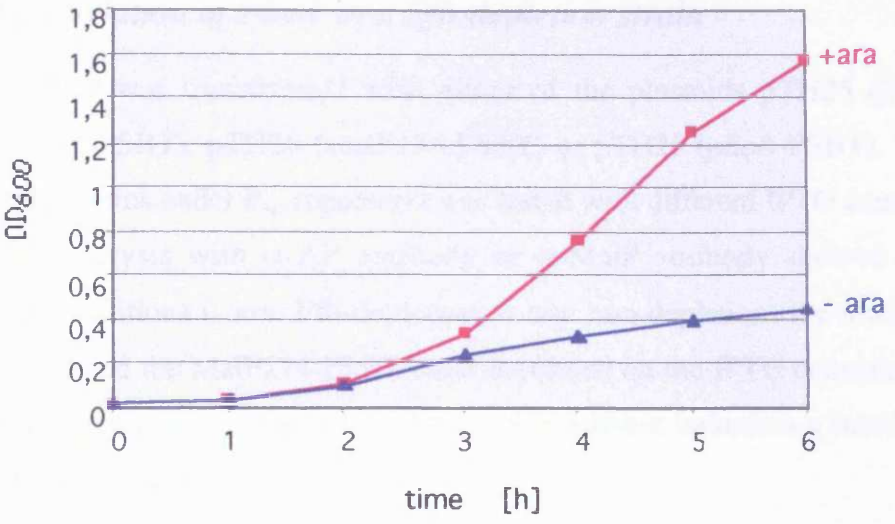


Fig. 4.57 Growth of WAM113 in NZA medium with 100 µg/ml kanamycin and 0.2% arabinose or without arabinose (Ffh-depletion). An overnight culture grown in NZA medium with 100 µg/ml kanamycin and 0.2% arabinose was washed 3 times in arabinose-free NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. OD was measured every hour at a wavelength of 600 nm.

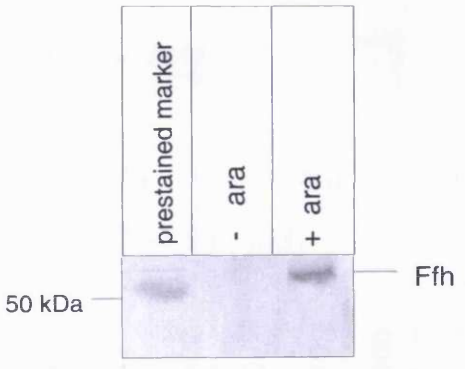


Fig. 4.58 Western blot from a 10% SDS-PAGE with α-Ffh antibody. WAM113 was grown in NZA medium with 100 µg/ml kanamycin and 0.2% arabinose, washed three times in arabinose-free medium and diluted 1/250 in NZA kanamycin medium with 0.2% arabinose to induce expression of Ffh, and in arabinose-free medium to deplete Ffh. Samples were taken after 3 hours of growth.

4.2.2.4 *Biotinylation of PSBT in a Ffh-depletion strain*

Strain WAM113 was transformed with either of the plasmids pTH25 (*ftsQ*-PSBT), pTH27 (*malF66*-PSBT), pTH28 (*malF*274-PSBT) or pTH29 (*phoA*-PSBT). Expression of the hybrid proteins under P_{tac} regulation was tested with different IPTG concentrations. Western blot analysis with α-AP antibody or α-MalF antibody showed that under experimental conditions (- ara: Ffh-depletion; + ara: non-depletion) the intensity of the AP-PSBT band and the MalF274-PSBT band depended on the IPTG concentration (Fig. 4.59). The *tac* promoter is not totally tight, as even without induction a small amount of the hybrid proteins was made.

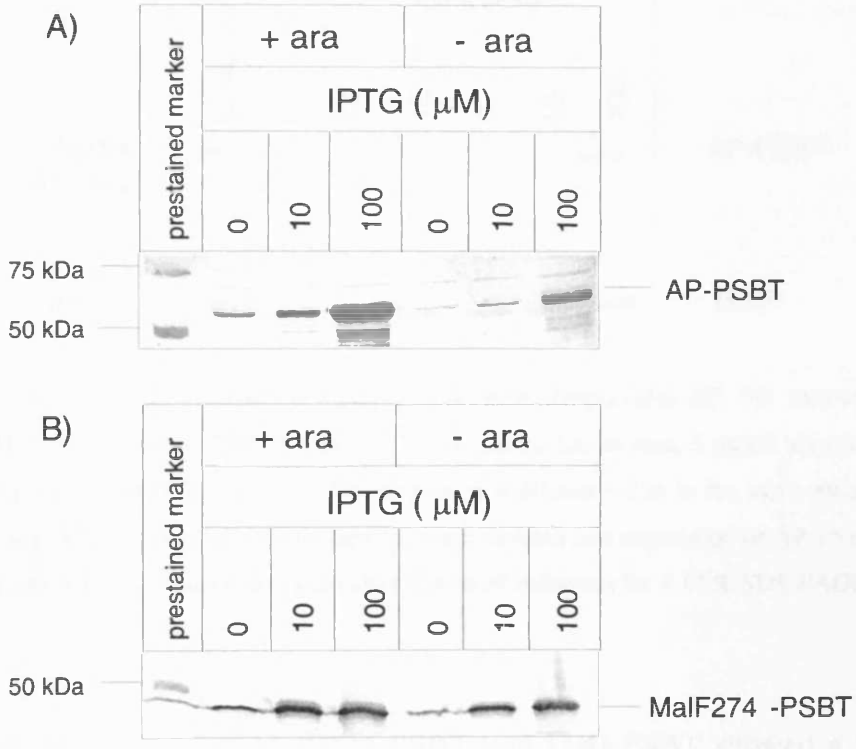


Fig. 4.59 Expression of AP-PSPT and MalF274-PSBT. Western blot from a 10% SDS-PAGE with A) α-AP antibody and B) α-MalF antibody. An overnight culture of WAM113 pTH29 and WAM113 pTH28 was grown in NZA medium with 100μg/ ml kanamycin, 5 μg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. After 3 hours of growth samples were divided and expression of AP-PSBT or MalF274-PSBT was induced with 10 or 100 μM IPTG or not induced at all. Samples were taken after 1 hour of induction.

The following biotinylation assays were performed with the same samples that were used for the Western blots showing the expression of the hybrid proteins. The AP-PSBT protein was biotinylated under depletion conditions only when expression was induced with 100 µM IPTG (Fig. 4.60). Some biotinylated protein was also detectable in the none-depleted control strain when this IPTG concentration was used. This result suggests that when high amounts of AP-PSBT are expressed the translocation process is slowed down. The higher intensity of the band in the depleted strain however indicates that Ffh-depletion has an effect on AP translocation.

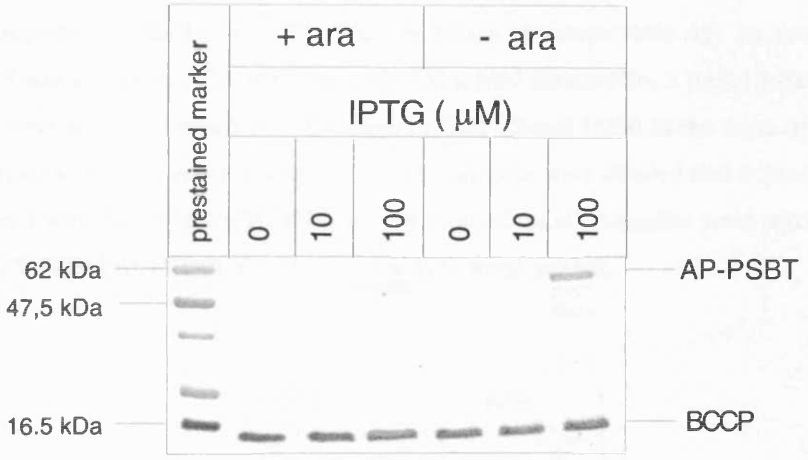


Fig. 4.60 Biotinylation of AP-PSBT. Western blot with streptavidin-AP. An overnight culture of WAM113 pTH29 was grown in NZA medium with 100 µg/ml kanamycin, 5 µg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% ara or without ara. After 3 hours of growth samples were divided and expression of AP-PSBT was induced with 10 or 100 µM IPTG. Samples were taken after 1 hour of induction for a 12 % SDS-PAGE.

The two membrane proteins MalF274-PSBT and FtsQ-PSBT showed a very similar biotinylation pattern (Fig. 4.61 and Fig. 4.62). A small amount of protein was biotinylated under none-depletion conditions when expression of the hybrid protein was induced with 100 µM IPTG. Under Ffh-depletion the amount of biotinylated protein increased with higher concentrations of inducer. In addition, even without induction biotinylated hybrid protein was detectable. The cytoplasmic control protein MalF66-PSPT was biotinylated under all conditions (result not shown).

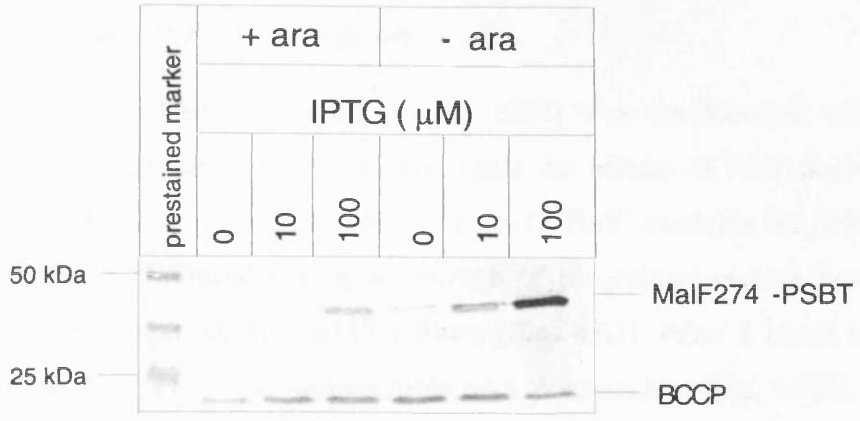


Fig. 4.61 Biotinylation of MalF274-PSBT. Western blot with streptavidin-AP. An overnight culture of WAM113 pTH28 was grown in NZA medium with 100 μg/ml kanamycin, 5 μg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. After 3 hours of growth samples were divided and expression of MalF274-PSBT was induced with 10 or 100 μM IPTG or not induced at all. Samples were taken after 1 hour of induction for a 12% SDS-PAGE. BCCP = biotin carboxyl carrier protein.

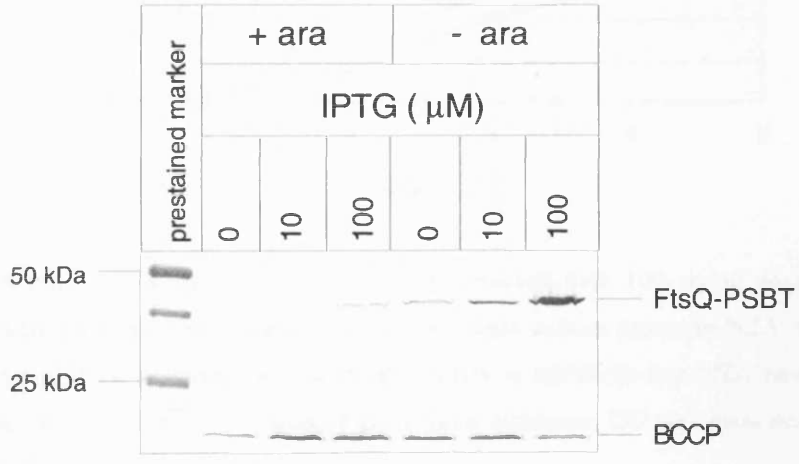


Fig. 4.62 Biotinylation of FtsQ-PSBT. Western blot developed with streptavidin-AP. An overnight culture of WAM113 pTH25 was grown in NZA medium with 100 μg/ml kanamycin, 5 μg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. After 3 hours of growth samples were divided and expression of FtsQ-PSBT was induced with 10 or 100 μM IPTG or not induced at all. Samples were taken after 1 hour of induction for a 12% SDS-PAGE. BCCP = biotin carboxyl carrier protein.

4.2.2.5 *Conditions of FtsY-depletion*

Strain N4156::pAra14-FtsY' (Luirink *et al.*, 1994) was transformed with plasmids pTH25, pTH27, pTH28 and pTH29 to investigate the effects of FtsY-depletion on the translocation of AP, FtsQ and MalF. N4156::pAra14-FtsY' contains the *ftsY* gene under the control of P_{araBAD}. Without arabinose growth of the culture ceased, however, more slowly than the Ffh-depleted WAM113 culture (Fig. 4.63). After 3 hours of growth in arabinose-free medium FtsY was undetectable on a Western blot (Fig. 4.64).

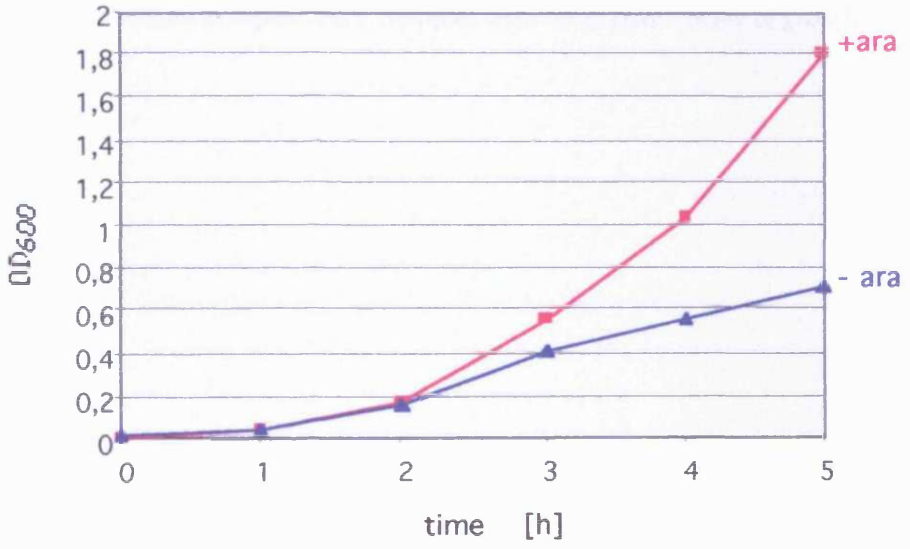


Fig. 4.63 Growth of N4156::pAra14-FtsY' in NZA medium with 100 µg/ml ampicillin and 0.2% arabinose or without arabinose (FtsY-depletion). An overnight culture grown in NZA medium with 100 µg/ml kanamycin and 0.2% arabinose was washed 3 times in arabinose-free NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. OD was measured every hour at a wavelength of 600 nm

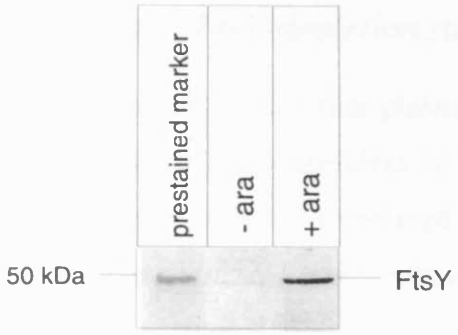


Fig. 4.64 Western blot from a 10% SDS-PAGE with α-FtsY antibody. N4156::pAra14-FtsY' was grown in NZA medium with 100 µg/ml ampicillin and 0.2% arabinose, washed three times in arabinose free medium and diluted 1/250 in NZA kanamycin medium with 0.2% arabinose to induce expression of FtsY, and in arabinose-free medium to deplete FtsY. Samples were taken after 3 hours of growth.

4.2.2.6 *Biotinylation of PSBT in a FtsY-depletion strain*

Strain N4156::pAra14-FtsY' transformed with either plasmid pTH28 or pTH29 showed expression of MalF274-PSBT and AP-PSBT, dependent on the IPTG concentration (Fig. 4.65). As expected, the hybrid proteins were expressed under depletion and none-depletion conditions. A small amount of protein was synthesised even without induction.

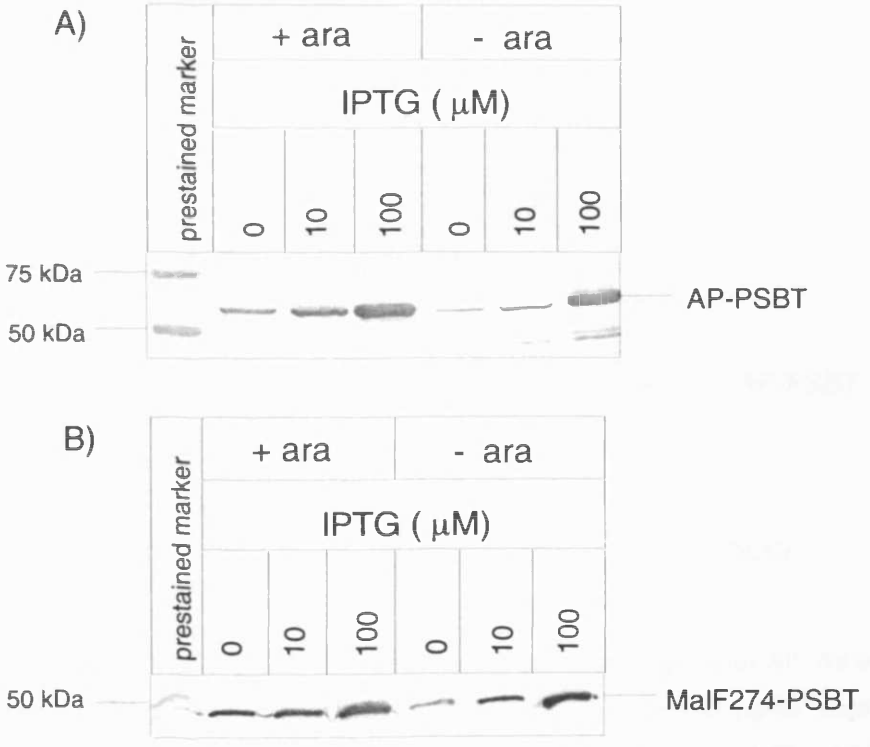


Fig. 4.65 Expression of AP-PSBT and MalF274-PSBT. Western blot from a 10% SDS-PAGE with A) α-AP antibody and B) α-MalF antibody. An overnight culture of N4156::pAra14-FtsY' pTH28 or pTH29 was grown in NZA medium with 100 µg/ml ampicillin, 5 µg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. After 3 hours of growth samples were divided and expression of the PSBT hybrid proteins was induced with 10 or 100 µM IPTG or not induced at all. Samples for SDS-PAGE were taken 1 hour after induction.

Biotinylation patterns of AP-PSBT, MalF274-PSBT and FtsQ-PSBT in strain N4156::pAra14-FtsY' were very similar to those observed under Ffh-depletion. Again, an effect on translocation of the secretory protein AP was detectable when FtsY was depleted and when protein expression was induced with 100 µM IPTG (Fig. 4.66). In contrast to Ffh-depletion a slight biotinylation of AP was also detected under FtsY-depletion when the protein was induced with only 10 µM IPTG. A slight biotinylation of AP-PSBT was also detected in the none-depleted cells. Part of this effect might be due to the overexpression of the hybrid protein.

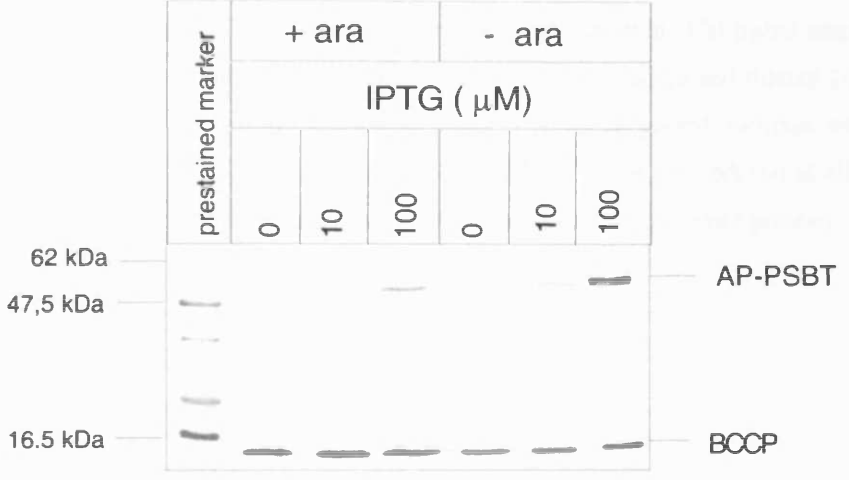


Fig. 4.66 Biotinylation of AP-PSBT. Western blot developed with streptavidin-AP. An overnight culture of N4156::pAra14-FtsY' pTH29 was grown in NZA medium with 100 µg/ml ampicillin, 5 µg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2 % arabinose or without arabinose. After 3 hours of growth samples were divided and expression of the AP-PSBT hybrid protein was induced with 10 or 100 µM IPTG or not induced at all. Samples for a 12% SDS-PAGE were taken 1 hour after induction. BCCP = biotin carboxyl carrier protein.

When MalF274-PSBT and FtsQ-PSBT were overexpressed with 100 µM IPTG under none-depletion conditions, also a small amount of biotinylated protein was visible on the respective Western blots (Fig. 4.67 and 4.68). As observed under Ffh-depletion, depletion of FtsY caused biotinylation of the PSBT domain of both membrane proteins. Even when expression of the hybrid proteins was not induced a biotinylation band was detectable on the blots.

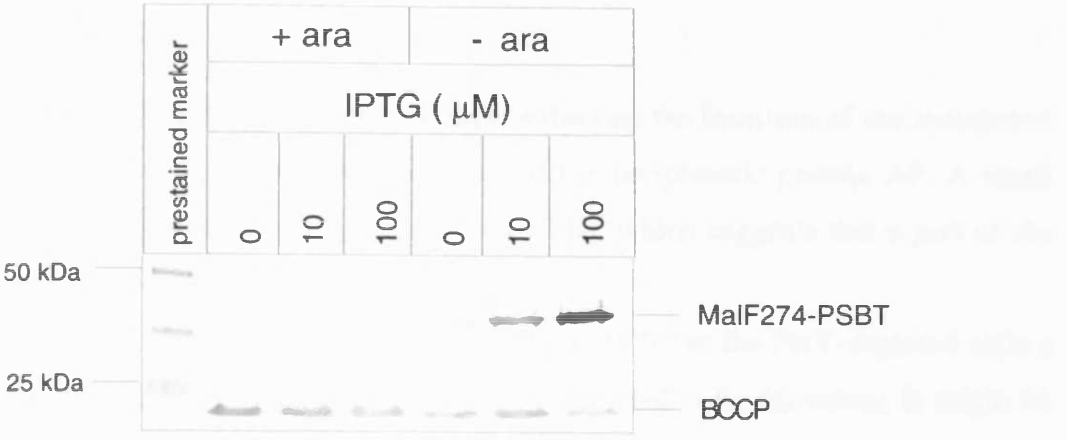


Fig. 4.67 Biotinylation of MalF274-PSBT. Western blot developed with streptavidin-AP. An overnight culture of N4156::pAra14-FtsY' pTH28 was grown in NZA medium with 100 μg/ml ampicillin, 5 μg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. After 3 hours of growth samples were divided and expression of MalF274-PSBT was induced with 10 or 100 μM IPTG or not induced at all. Samples for a 12% SDS-PAGE were taken 1 hour after induction. BCCP = biotin carboxyl carrier protein.

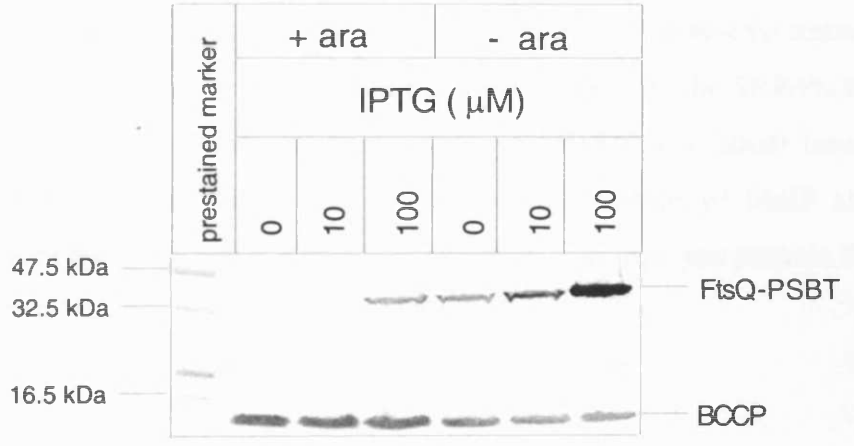


Fig. 4.68 Biotinylation of FtsQ-PSBT. Western blot developed with streptavidin-AP. An overnight culture of N4156::pAra14-FtsY' pTH25 was grown in NZA medium with 100 μg/ml ampicillin, 5 μg/ml tetracycline and 0.2% arabinose. Cells were washed 3 times in NZA medium and diluted 1/250 in the same medium with 0.2% arabinose or without arabinose. After 3 hours of growth samples were divided and expression of FtsQ-PSBT was induced with 10 or 100 μM IPTG or not induced at all. Samples for a 12% SDS-PAGE were taken 1 hour after induction. BCCP = biotin carboxyl carrier protein.

4.2.3 Conclusions

Depletion of Ffh and FtsY had a much stronger effect on the insertion of the membrane proteins MalF274 and FtsQ than the secretion of the periplasmic protein AP. A small amount of AP-PSBT accumulated in undepleted cells, which suggests that a part of the effect was due to overproduction of AP-PSBT.

When AP-PSBT expression was induced with 10 μM IPTG in the FtsY-depleted cells a biotinylation effect was visible in contrast to Ffh-depleted cells. However, it might be that this effect was a result of the reported negative effect of FtsY-depletion on SecY expression (Herskovits and Bibi, 2000).

Depletion of SecA830 had an impact not only on the secretion of AP-PSBT but also on the insertion of the MalF fragment 274 and the small membrane protein FtsQ.

Similar results were observed with the SecA195 inactivation strains TH47 pTH10 and TH27 pTH9. The biotinylation effect was stronger when SecA195 was proteolysed by the ribosome-attached TEV protease (at least on AP secretion and insertion of MalF274).

These results indicate that SecA plays a role in the insertion of at least some cytoplasmic membrane proteins that are targeted to the inner membrane via the SRP/FtsY pathway. In addition, not only large membrane proteins but probably also small inner membrane proteins are substrates of SecA. However, a shared feature of MalF and FtsQ that probably contributes to the SecA-dependent insertion is a large periplasmic domain.

4.3 Changes in gene expression under Ffh-, FtsY- or SecA-depletion

Ffh, the proteineous component of the SRP and its receptor FtsY are like SecA essential for the viability of the cell. The assumed function of these proteins is to target nascent, ribosome-associated polypeptides to the membrane for subsequent insertion. However, it is not clear if additional components are involved in the targeting process or if FtsY or Ffh might fulfil other essential roles in the cell. Also, not much is known about regulatory pathways concerning Ffh and FtsY expression or the existence of possible alternative targeting routes or factors. To address these questions the global gene response was studied in cells that were depleted of either Ffh, FtsY or SecA. The recently developed DNA-array technology based on the completed *E. coli* genome project (Blattner *et al.*, 1997) provided a strong approach to study transcriptional expression patterns. Transcriptional gene expression profiles were obtained by hybridising ^{33}P-labelled cDNA onto macroarrays containing PCR-amplified DNA from all 4290 ORFs of *E. coli* (Sigma Genosys). The cDNA was prepared from RNA extracted from cells expressing Ffh, FtsY and SecA, respectively, and from cells where either of these proteins were depleted. Hybridised probes were visualised on image files using autoradiography and PhosphorImager Screens. The generated data were statistically analysed as described in chapter 3.6.9.5, using Significance Analysis of Microarrays (SAM) developed by Tusher and coworkers (Tusher *et al.*, 2001). This method is based on gene-specific t-tests and permutation analysis. A score is calculated for each gene based on its change in gene expression relative to the standard deviation of repeated measurements. Genes that score greater than a cutoff threshold are considered potentially significant. The threshold is freely adjustable to identify smaller or larger sets of genes and a false discovery rate (the percentage of genes that are identified by chance) is calculated for each set.
Western blot analysis was performed to confirm and compare results on the protein level for genes that were identified as being significantly modulated by depletion.

4.3.1 Gene expression profile of Ffh-depleted cells

Ffh was depleted in strain WAM113 as described in chapter 4.2.2.3 in the absence of the inducer arabinose. Cells for RNA extraction were harvested during early to mid logarithmic growth phase after 3 hours of depletion. The average OD_{600} at the time of sampling was 0.33 for the arabinose induced control culture and 0.23 for the depleted test culture.

4.3.1.1 Transcriptional analysis of gene expression

A basic statistical analysis of the data generated significant changes in gene expression for six genes. Ratios outside a threshold of two standard deviations from the mean were considered to be significant with 95% confidence. According to this method genes *dnaK*, *hslS* and *hslT* were up-regulated under Ffh-depletion whereas *fliC*, *rplL* and *cspA* were down-regulated. A visual comparison of the pictures of the depleted and undepleted array membranes as illustrated in Fig. 4.69 confirmed this result.

Nevertheless, this method was unsatisfactory as it was not sensitive enough and did not take into account the changes referring to every individual gene. Therefore, the data set was analysed again using an acknowledged method developed for the statistical analysis of microarrays (SAM) that could also be applied for the macroarray data here. A prerequisit for the statistical analysis is the normal distribution of the data. The gene expression ratios for each replicate were plotted in histograms (Appendix chapter B.a, Fig. B.1) to test for normal distribution. For each gene a score d_i, also called relative difference, was calculated and plotted against the expected relative difference d_{iE} (Fig. 4.70). Genes that were represented by points displaced from the $d_i \approx d_{iE}$ line by a distance greater than a threshold Δ were called significant. The confidence level on which a gene is called significant here is dependent on the chosen Δ value. For each Δ the program calculates a q-value, which is the lowest false discovery rate at which a gene is called significant. When Δ was 0.178 a set of 28 significantly modulated genes was generated, including those six genes identified earlier with a FDR of 21.4%. With a Δ value of 0.379 only 17 genes are called significant but with a much better FDR of 5.9%, which corresponds to a confidence value of about 94%.

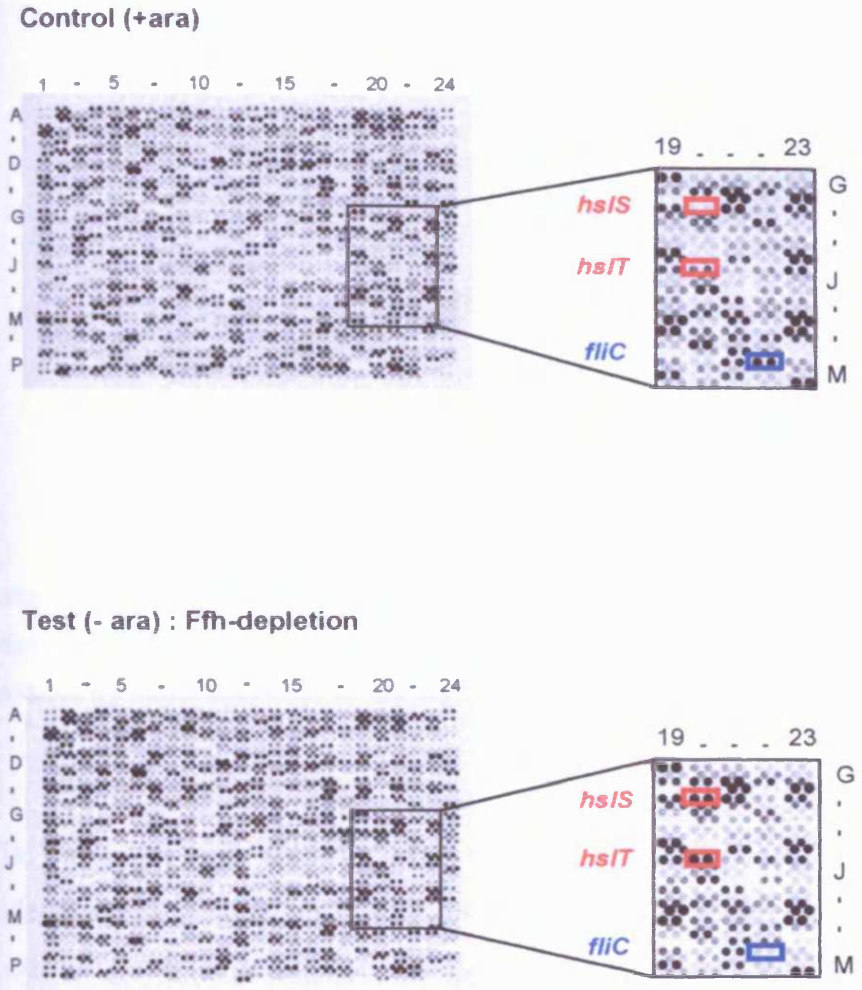


Fig. 4.69 Detail of DNA array membranes of Ffh-induced (control) and Ffh-depleted (test) probes after phosphorimaging. For each probe only field 1 of the array membrane is shown. Fields 2 and 3 are not shown but structured in a similar manner. Each field is divided in a primary grid: columns 1 to 24 and rows A to P. The secondary grids usually contain 4 ORFs spotted in duplicates. The four corners of a field contain four spots of *E. coli* genomic DNA as a positive control. The visible changes in gene expression comparing control and test arrays for some of the significantly modulated genes are shown in the enlarged boxes on the right. Up-regulated genes are marked in red, down-regulated genes are marked in blue.

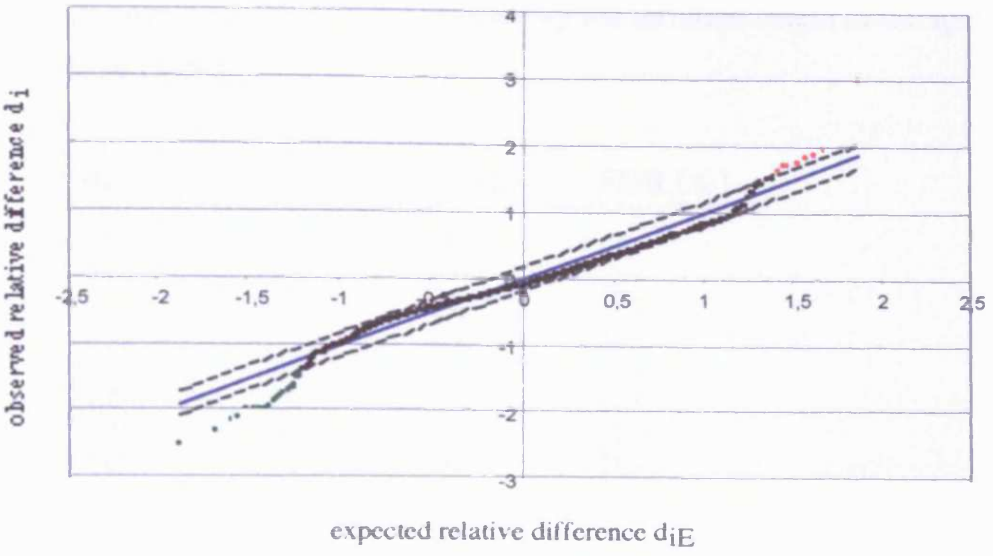


Fig. 4.70 Scatter plot of the observed relative difference d_i versus the expected relative difference d_{iE}. Data derived from the Ffh-depletion experiment. The blue solid line indicates the $d_i = d_{iE}$ line. The dotted lines indicate the cutoff threshold at a distance of $\Delta = 0.178$ from the solid line.

In Table 4.1 and Table 4.2 the up- and down-regulated genes are listed that were identified as being significantly modulated on the basis of a Δ value of 0.178. The FDR value is given for each gene. The genes are sorted according to their d_i value, which is the t-test score. Genes identified as significant by d_i do not necessarily have the largest change in gene expression. The average fold change of gene expression r_i (test/control) is the numerator of the t-test equation and is given in $\log_2$ transformation. A r_i value of 1 means a gene induction of two-fold (r_i = -1 corresponds to a two-fold repression). The gene *arsB* has a higher d_i value than *hslT* (Tab. 4.1). However, the r_i value for *hslT* of 1.43 means a 2.7-fold up-regulation of gene expression, whereas a r_i value for *arsB* of 1.11 means only a 2.16-fold up-regulation.

A small change in gene expression of certain genes, for example genes encoding transcription regulators, can have a major effect on the cell status. Therefore, the magnitude of fold difference in gene expression does not correlate with the importance these changes have for the cell. Also, a direct comparison of expression levels of genes can be flawed as the efficiency of reverse transcriptase reaction might vary for different

RNAs and hybridising intensities are affected by the different length of the spotted DNAs (Richmond *et al.*, 1999).

Gene	d_i	r_i	$s_i + s_0$	FDR [%]
hslS	3.01	1.64	0.55	5.9
dnak	1.98	1.49	0.75	12.5
yhcN	1.88	0.74	0.39	12.5
arsB	1.83	1.11	0.60	12.5
hslT	1.77	1.43	0.81	12.5
glpD	1.74	1.30	0.74	12.5
ynaJ	1.71	1.02	0.60	12.5
clpB	1.65	1.06	0.64	21.4

Tab. 4.1 Genes identified by SAM as potentially significantly up-regulated when Ffh was depleted in WAM113. Data was derived from 5 independent replicates. d_i is the t-test score, r_i the average gene expression ratio ($\log_2$ transformed) and ($s_i + s_0$) the denominator of the t-test equation is a measure of the error of the data.

Gene	d_i	r_i	$s_i + s_0$	FDR [%]
fliC	-2.48	-2.35	0.95	5.9
rplL	-2.29	-1.20	0.53	5.9
araA	-2.16	-1.35	0.62	5.9
tktA	-2.10	-0.73	0.35	5.9
tufB	-1.96	-0.83	0.42	5.9
lysU	-1.96	-0.74	0.38	5.9
ftsN	-1.96	-0.83	0.42	5.9
rplJ	-1.96	-0.96	0.49	5.9
ompA	-1.95	-1.39	0.72	5.9
pqk	-1.85	-0.74	0.40	5.9
fliA	-1.80	-0.87	0.49	5.9
fba	-1.77	-0.71	0.40	5.9
flgE	-1.75	-1.09	0.62	5.9
tsr	-1.67	-0.63	0.38	5.9
flgF	-1.66	-0.59	0.35	5.9
flgB	-1.66	-0.76	0.46	5.9
narG	-1.61	-0.81	0.51	12.5
flgG	-1.48	-0.71	0.48	21.4
cspA	-1.48	-1.34	0.90	21.4
eno	-1.45	-0.64	0.44	21.4

Tab. 4.2 Genes identified by SAM as potentially significantly down-regulated when Ffh was depleted in WAM113. Data was derived from 5 independent replicates. d_i is the t-test score, r_i the average gene expression ratio ($\log_2$ transformed) and ($s_i + s_0$) the denominator of the t-test equation is a measure of the error of the data.

A classification of the gene products of the significantly modulated genes is listed in the chapter B.b. (see Appendix). Most up-regulated genes belong to the group of heat shock response genes (Appendix, Tab. B.1). The bacterial Hsp70 chaperone homolog DnaK plays an important role in stabilising extended polypeptide segments (Gross, 1996), whereas the function of the two small heat shock proteins HslS and HslT is not yet clearly defined. ClpB is a heat inducible subunit of an intracellular ATP-dependent protease (Kitagawa *et al.*, 1991).

Two other major functional groups were affected by Ffh-depletion. One group was the group of the flagellar assembly genes. Several genes encoding flagellar hook and rod proteins were down-regulated. In addition, genes *fliA* and *fliC* were identified as negatively regulated genes. *fliA* encodes the RNA sigma factor for a flagellar operon, and *fliC* encodes the constituent of the flagellar filament, flagellin. The other negatively affected group was the group of genes encoding ribosomal proteins of the large subunit (*rplL*, *rplJ*) or other proteins involved in translation.

4.3.1.2 Translational analysis of gene expression

A Western blot analysis was performed to confirm the results obtained by the DNA array experiments as well as to detect possible differences in regulation on the translational level (Fig. 4.71). Without arabinose in the medium no Ffh was detected in WAM113 after 3 hours of growth, whereas the amount of FtsY was unaffected. All tested heat shock proteins showed increased amounts of protein level upon depletion of Ffh confirming the result of the DNA array on the mRNA level. It has to be noted that HslS and HslT have the same mobility in SDS-PAGE and are indistinguishable by polyclonal antibodies because they share 48% of identical amino acid sequence (Chuang *et al.*, 1993). Although the antibody used was raised against HslS, it might not be possible to tell to what amount both proteins contribute to the detected band. Expression of SecA remained unaffected by Ffh-depletion on transcriptional and translational level.

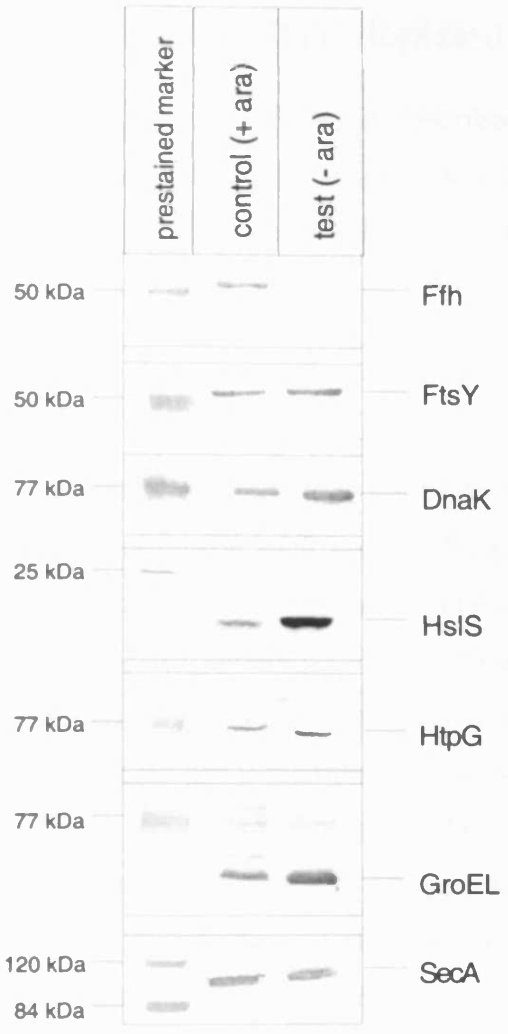


Fig. 4.71 Western blot analysis of selected proteins with the stated antibodies. An overnight culture of strain WAM113 was grown in NZA medium with 0.2% arabinose and 100 µg/ml kanamycin, washed three times in arabinose free medium and diluted 1/250 in NZA medium with 0.2% arabinose (control) to induce Ffh expression or in arabinose free NZA medium (test) for Ffh-depletion. Samples for 10% SDS-PAGE were taken after 3 hours of growth.

4.3.2 Gene expression profile of FtsY-depleted cells

FtsY was depleted in strain N4156::pAra14-FtsY' as described in chapter 4.1.1.3. Cells for RNA extraction were taken after 3 hours of growth in NZA medium with 0.2% arabinose or without the inducer at an OD_{600} of about 0.55 for the control culture and 0.4 for the test culture.

4.3.2.1 Transcriptional analysis of gene expression

A first statistical analysis of the DNA-array data of the FtsY-depleted versus undepleted cells showed the significant up-regulation of 9 genes: *dnaK*, *hslS*, *hslT*, *mopB*, *glpD*, *nanA*, *nanT*, *yjhB* and *yjhC*. Four independent replicates were analysed using the two-fold standard deviation of each replicate as a threshold for significance with 95% confidence. Changes in gene expression of these genes were also confirmed by comparing the scanned pictures of the test and control array membranes (Fig. 4.72).

However, as with the Ffh DNA-array data above, the DNA-array data of the FtsY-depletion experiment was also analysed using SAM. The scatter plot of the SAM analysis (Fig. 4.73) showed an asymmetric distribution, as there are more genes significantly up-regulated than down-regulated when FtsY was depleted.

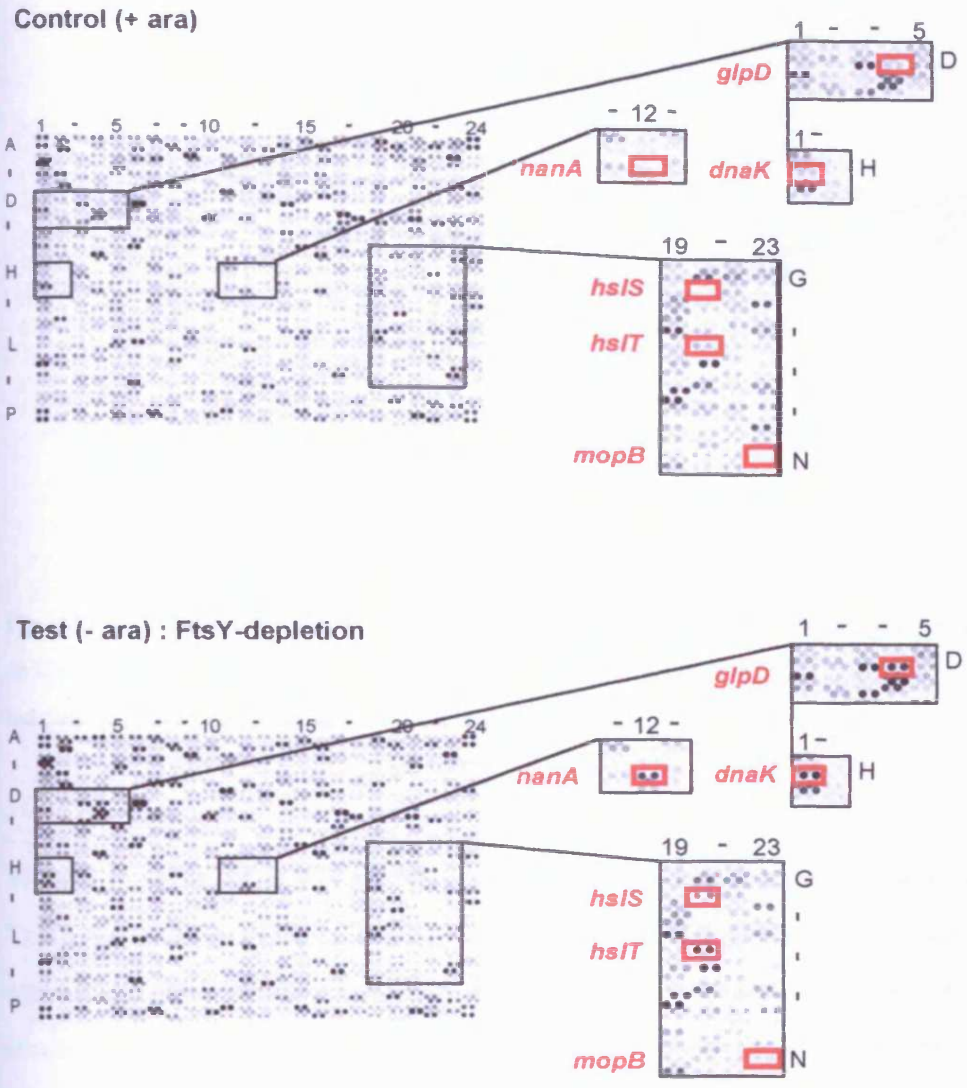


Fig. 4.72 Detail of DNA array membranes of FtsY-induced (control) and FtsY-depleted (test) probes after phosphorimaging. For each probe only field 1 of the array membrane is shown. Fields 2 and 3 are not shown. Each field is divided in a primary grid: columns 1 to 24 and rows A to P. The secondary grids usually contain 4 ORFs spotted in duplicates. The four corners of a field contain four spots of *E. coli* genomic DNA as a positive control. The visible changes in gene expression comparing control and test arrays for some of the significantly modulated genes are shown in the enlarged boxes on the right. Up-regulated genes are marked in red.

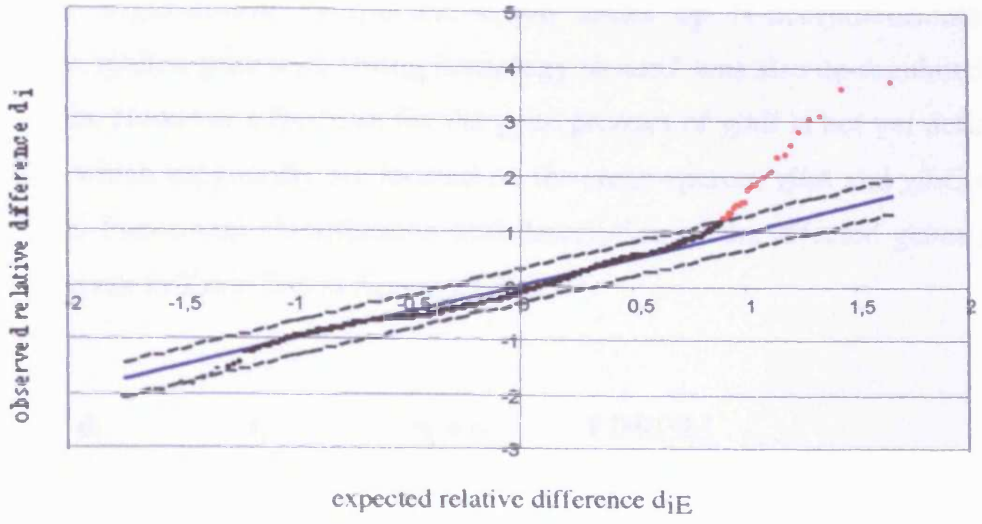


Fig. 4.73 Scatter plot of the observed relative difference d_i versus the expected relative difference d_{iE}. Data derived from the FtsY-depletion experiment. The solid line indicates the line for $d_i = d_{iE}$. The dotted lines indicate the cutoff threshold at a distance of $\Delta = 0.306$ from the solid line.

With a Δ value of 0.306 SAM generated 38 significant genes listed in Table 4.4 and Table 4.5 with a FDR of 6.5%. A lower FDR of 3.4% was calculated for 24 positive significant genes when a cutoff of $\Delta = 0.457$ was chosen. In contrast to the Ffh depleted strain the flagellar operon was hardly affected. However, the effect of FtsY-depletion on the expression of genes belonging to the heat shock response was much more pronounced than in the case of Ffh-depletion. 9 genes encoding heat inducible chaperones and proteases were significantly up-regulated. Apart from genes *hslS/T*, *dnaK* and *clpB* also genes *mopA/mopB* and *htpG* encoding the heat shock chaperones GroEL/GroES (Kusukawa and Yura, 1988) and HtpG (Heitzer *et al.*, 1990), as well as the genes encoding the heat inducible proteases HslV and HtpX (Soel *et al.*, 1997; Kornitzer *et al.*, 1991) were positively regulated.

The N-acetylneuraminate lyase encoded by the up-regulated *nanA* gene converts N-acetylneuraminic acid into pyruvate and N-acetylmannosamine, which is then converted in several steps to a precursor of cell wall components (Plumbridge and Vimr, 1999). Another gene from the *nan*-operon, which showed increased expression was *nanT*,

encoding a sugar-cation symporter which takes up N-acetylneuraminic acid. Interestingly, *yjhB*, a gene with strong homology to *nanT* was also up-regulated in FtsY-depleted cells. However a function for the gene product of *yjhB* is not yet defined. Two more genes which supposedly are located on the same operon, *yjhA* and *yjhC* were also up-regulated. Functional classification and description of the affected genes and gene products is given in Table B.2 in Appendix B.b.

Gene	d_i	r_i	$s_i + s_0$	FDR[%]
yjhC	3.75	4.25	1.13	3.4
hslS	3.58	3.52	0.98	3.4
mopB	3.12	2.50	0.80	3.4
yjhB	3.06	2.55	0.83	3.4
nanA	2.82	2.74	0.97	3.4
nanT	2.59	1.90	0.73	3.4
hslT	2.39	2.08	0.87	3.4
mopA	2.33	1.54	0.66	3.4
yjhA	2.09	1.73	0.83	3.4
tnaA	2.02	2.17	1.07	3.4
dnak	1.98	2.18	1.10	3.4
rpiB	1.91	1.23	0.64	3.4
glpD	1.86	2.05	1.11	3.4
glpK	1.82	1.33	0.73	3.4
htpG	1.82	1.09	0.60	3.4
tnaL	1.80	1.67	0.93	3.4
yhcI	1.75	0.90	0.52	3.4
hslV	1.52	1.10	0.72	3.4
yhcH	1.49	1.20	0.80	3.4
yacG	1.49	1.22	0.82	3.4
cspE	1.48	0.94	0.63	3.4
gltA	1.45	1.06	0.73	3.4
sucD	1.41	1.18	0.83	3.4
clpB	1.40	1.26	0.90	3.4
yaiW	1.33	0.84	0.63	6.4
sdhB	1.32	0.72	0.54	6.4
hflB	1.31	0.77	0.59	6.4
gatA	1.31	0.96	0.73	6.4
cynT	1.27	1.45	1.14	6.5
nikD	1.23	0.68	0.55	6.5
narY	1.23	0.90	0.73	6.5
htpX	1.22	0.73	0.60	6.5

Tab. 4.4 Genes identified by SAM as potentially significantly up-regulated when FtsY was depleted in N4156::pAra14-FtsY'. Data was derived from 4 independent replicates. d_i is the t-test score, r_i the average gene expression ratio ($\log_2$ transformed) and ($s_i + s_0$) the denominator of the t-test equation is a measure of the error of the data.

Only a few genes were significantly down-regulated upon FtsY-depletion, most of which are genes encoding ribosomal proteins (Tab. 4.5).

Gene	d_i	r_i	$s_i + s_0$	FDR[%]
rplI	-2.05	-0.94	0.46	6.4
rplL	-1.96	-2.05	1.04	6.4
ompX	-1.83	-1.62	0.89	6.4
rplJ	-1.80	-1.01	0.56	6.4
trmD	-1.73	-1.18	0.68	6.5
lysU	-1.69	-0.95	0.56	6.5

Tab. 4.5 Genes identified by SAM as potentially significantly down-regulated when FtsY was depleted in N4156::pAra14-FtsY'. Data was derived from 4 independent replicates. d_i is the t-test score, r_i the average gene expression ratio (log_2 transformed) and ($s_i + s_0$) the denominator of the t-test equation is a measure of the error of the data.

4.3.2.2 Translational analysis of gene expression

In arabinose free medium hardly any FtsY was detected in strain N4156::pAra14-FtsY' after 3 hours of growth whereas there was no difference in Ffh and SecA levels (Fig. 4.74). The result of the DNA array was confirmed as all examined heat shock chaperones showed increased amounts of protein upon FtsY-depletion.

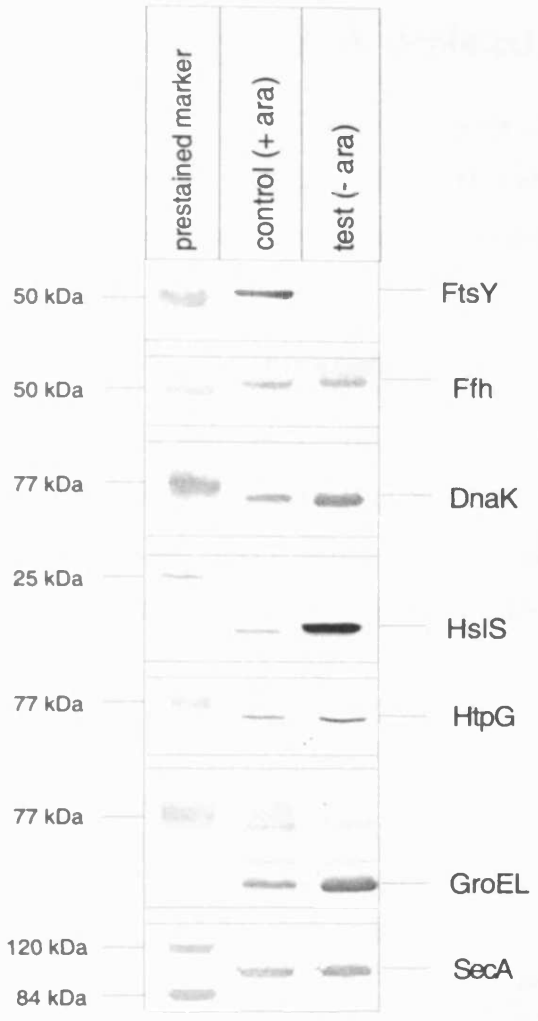


Fig. 4.74 Western blot analysis of selected proteins with the stated antibodies. An overnight culture of strain N4156::pAra14-FtsY' was grown in NZA medium with 0.2% arabinose and 100 µg/ml ampicillin, washed three times in arabinose free medium and diluted 1/250 in NZA medium with 0.2% arabinose (control) to induce FtsY expression or in arabinose free NZA medium (test) for FtsY-depletion. Samples for 10% SDS-PAGE were taken after 3 hours of growth.

4.3.3 Gene expression profile of SecA-depleted cells

SecA830 was depleted in strain TH25 pTHS19 in the absence of the inducer IPTG. Control cultures were grown in NZA medium with 100 µM IPTG. Cells for RNA extraction and Western blot were taken during early to mid-logarithmic phase.

4.3.3.1 Transcriptional analysis of gene expression

Statistical analysis of the changes in gene expression ratios of the SecA-depleted versus undepleted cells by SAM generated 161 significantly modulated genes with a Δ value of 0.703 and a false discovery rate (FDR) of 6% (Fig. 4.75). With a lower Δ value the FDR decreased to 2.3%, and still gene expression of 45 genes changed significantly (Tab. 4.6 and 4.7).

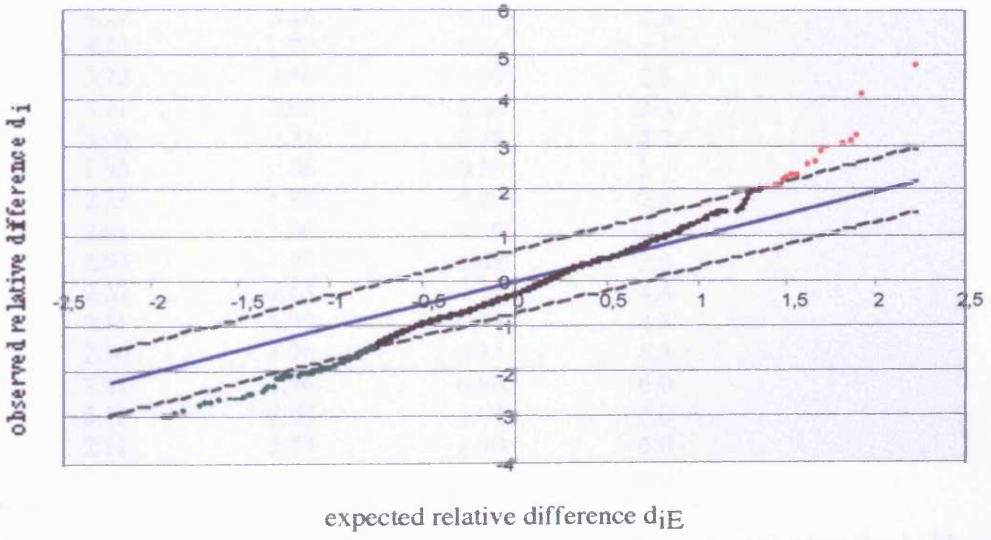


Fig. 4.75 Scatter plot of the observed relative difference d_i versus the expected relative difference d_{iE}. Data derived from the SecA830-depletion experiment. The solid line indicates the line for $d_i = d_{iE}$. The dotted lines indicate the cutoff threshold at a distance of $\Delta = 0.703$ from the solid line.

Detailed information about the affected genes and the classification of the gene products according to their function is given in Tab. B.3 (Appendix). Among the 16 genes that

were significantly up-regulated was the complete phage shock operon *pspABCDE* (Tab. 4.6). Gene expression of 145 genes was negatively affected by SecA-depletion with a maximal FDR of 6% (Tab. 4.7). Genes that encode integral membrane proteins belonging to various transport systems formed the biggest group of genes that were significantly down-regulated. Another group of down-regulated genes encode integral membrane proteins that are involved in signal transduction and transcription regulation. Some genes encoding periplasmic proteins were also negatively affected by SecA-depletion, e.g. *htrA*, encoding the periplasmic protease and chaperone DegP. SecA is an ATP-dependent helicase, at least 5 genes that encode other ATP-dependent helicases (*deaD*, *hrpB*, *recQ*, *recB*, *dnaB*) were down-regulated by SecA-depletion.

Gene	d_i	r_i	$s_i + s_0$	FDR[%]
pspB	4.77	3.18	0.67	2.3
pspE	4.12	2.72	0.66	2.3
pspD	3.22	3.50	1.09	2.3
msyB	3.11	2.06	0.66	2.3
glgS	3.06	2.38	0.78	2.3
pspC	2.93	2.86	0.98	2.3
hdeB	2.83	1.98	0.70	2.3
yijC	2.61	1.80	0.69	2.3
infC	2.54	1.73	0.68	2.3
adhE	2.32	1.65	0.71	4.3
yaiA	2.31	1.72	0.74	4.3
nlpD	2.30	1.96	0.85	4.3
rimL	2.22	1.96	0.88	6.0
ydcH	2.12	2.20	1.04	6.0
intE	2.11	2.74	1.30	6.0
pspA	2.07	2.68	1.29	6.0

Tab. 4.6 Genes identified by SAM as potentially significantly up-regulated when SecA830 was depleted in TH25 pTHS19. Data was derived from 2 independent replicates. d_i is the t-test score, r_i the average gene expression ratio ($\log_2$ transformed). The denominator of the t-test equation ($s_i + s_0$) is a measure of the error of the data.

Gene	d_i	r_i	$s_i + s_0$	FDR[%]
ygdQ	-2.98	-2.32	0.78	2.3
uxuB	-2.97	-2.69	0.90	2.3
agaS	-2.96	-1.97	0.67	2.3
yccS	-2.94	-2.22	0.76	2.3
yicE	-2.87	-2.03	0.71	2.3
gntT	-2.77	-2.23	0.81	2.3
gntP	-2.72	-2.67	0.98	2.3
yigN	-2.68	-2.12	0.79	2.3
nrfG	-2.63	-1.76	0.67	2.3
hydG	-2.60	-2.34	0.90	2.3
dsdX	-2.60	-2.70	1.04	2.3
mtr	-2.60	-3.00	1.16	2.3
mrdB	-2.54	-2.09	0.83	2.3
frdB	-2.49	-1.66	0.66	2.3
yjjP	-2.47	-1.75	0.71	2.3
fruA	-2.45	-1.73	0.71	2.3
sgcC	-2.37	-3.02	1.28	2.3
yaaF	-2.35	-1.73	0.74	2.3
treB	-2.31	-1.74	0.75	2.3
intR	-2.16	-1.59	0.73	2.3
yfhT	-2.16	-1.50	0.69	2.3
yfhK	-2.15	-1.73	0.80	2.3
dnaE	-2.13	-1.69	0.79	2.3
b0299	-2.11	-2.83	1.34	2.3
ascG	-2.11	-1.67	0.79	2.3
fucI	-2.10	-1.82	0.87	2.3
yhdX	-2.10	-2.37	1.13	2.3
fecE	-2.09	-1.68	0.80	2.3
amiA	-2.09	-1.42	0.68	2.3
rpsQ	-2.06	-1.50	0.73	2.3
ybjE	-2.06	-1.54	0.75	2.3
kdpD	-2.05	-1.99	0.97	2.3
yicC	-2.03	-1.60	0.79	2.3
ygfL	-2.03	-1.85	0.91	2.3
ybhE	-2.03	-1.34	0.66	2.3
mdoH	-2.02	-2.14	1.06	2.3
rfc	-2.00	-1.44	0.72	3.1
hydH	-2.00	-1.41	0.70	3.1
dmsA	-1.98	-2.74	1.38	3.1
yieO	-1.97	-1.73	0.88	3.1
htrA	-1.97	-1.60	0.81	3.1
serB	-1.96	-1.31	0.67	3.1
cysS	-1.95	-2.09	1.08	3.1
dppB	-1.95	-2.51	1.29	3.1
livG	-1.94	-1.94	1.00	3.1
deaD	-1.94	-1.60	0.83	3.1
yhdY	-1.93	-1.94	1.01	3.1
tdcC	-1.92	-2.33	1.21	3.1
yafH	-1.91	-1.31	0.68	3.1
yeiJ	-1.91	-2.72	1.43	3.1
ygiS	-1.91	-1.77	0.93	3.1
gntU_2	-1.90	-1.38	0.73	3.1
yqjG	-1.90	-1.99	1.05	3.1

yhgE	-1.90	-2.59	1.37	3.1
hrpB	-1.89	-1.85	0.98	3.1
rplI	-1.89	-2.28	1.20	3.1
cysQ	-1.87	-1.34	0.72	3.1
aceA	-1.87	-1.22	0.66	3.1
fhuC	-1.85	-1.42	0.77	3.3
bygiY	-1.85	-1.31	0.71	3.3
recQ	-1.84	-1.43	0.78	3.3
glyS	-1.83	-1.25	0.68	3.3
ybhC	-1.82	-1.58	0.87	3.3
mbhA	-1.81	-2.16	1.19	3.3
yfaX	-1.80	-2.20	1.22	4.3
nohB	-1.80	-2.23	1.24	4.3
yhdH	-1.79	-1.65	0.92	4.3
mppA	-1.78	-1.40	0.78	4.3
adi	-1.78	-2.97	1.67	4.3
pheS	-1.75	-1.27	0.72	4.3
pcnB	-1.75	-1.22	0.70	4.3
aroP	-1.74	-2.31	1.33	4.3
ycgE	-1.73	-1.32	0.76	4.3
chaC	-1.72	-1.16	0.67	4.3
yheB	-1.72	-2.45	1.43	4.3
hofG	-1.71	-1.57	0.92	4.3
yqgA	-1.71	-1.78	1.04	4.3
ydjF	-1.71	-1.32	0.77	4.3
rpsR	-1.70	-1.33	0.78	4.3
yiaV	-1.70	-2.41	1.42	4.3
yadC	-1.69	-2.16	1.27	4.3
yhjC	-1.69	-1.27	0.75	4.3
yjdE	-1.69	-2.68	1.59	4.3
ygbI	-1.69	-1.87	1.11	4.3
yfbS	-1.69	-2.40	1.42	4.3
sdaC	-1.68	-1.77	1.05	4.3
yhiV	-1.68	-2.56	1.52	4.3
gutQ	-1.67	-1.60	0.96	4.3
wzzE	-1.67	-1.12	0.67	4.3
yhhS	-1.67	-2.79	1.68	4.3
aqpZ	-1.66	-2.00	1.20	4.3
tra_1	-1.66	-1.84	1.11	4.3
yraJ	-1.66	-2.27	1.37	4.3
ybeK	-1.66	-1.42	0.86	4.3
yidJ	-1.66	-2.74	1.65	4.3
folK	-1.65	-1.16	0.70	4.3
ebqA	-1.65	-1.19	0.72	4.3
cmr	-1.65	-1.12	0.68	4.3
yhdW	-1.64	-1.83	1.12	4.3
priB	-1.63	-1.08	0.66	4.3
yabH	-1.62	-1.45	0.90	5.1
dnaB	-1.62	-1.48	0.91	5.1
rspA	-1.61	-1.40	0.87	5.1
nadC	-1.61	-1.77	1.10	5.1
ygcP	-1.61	-1.45	0.90	5.1
ygcS	-1.60	-2.54	1.58	5.1
yihA	-1.60	-1.06	0.66	5.1
ycbN	-1.60	-1.60	1.00	5.1
manX	-1.59	-1.21	0.76	5.1

ydcR	-1.59	-2.00	1.26	5.1
pepE	-1.58	-1.93	1.22	5.1
recG	-1.58	-1.72	1.09	6.0
bisZ	-1.58	-2.42	1.54	6.0
uxaC	-1.57	-1.93	1.23	6.0
pepB	-1.57	-1.47	0.93	6.0
yhaF	-1.57	-2.55	1.62	6.0
phnK	-1.56	-2.23	1.42	6.0
wzb	-1.56	-1.06	0.68	6.0
gltS	-1.55	-1.20	0.78	6.0
ycdJ	-1.54	-1.17	0.76	6.0
hybO	-1.54	-1.54	1.00	6.0
fucK	-1.54	-1.67	1.09	6.0
holD	-1.54	-1.62	1.05	6.0
hycG	-1.53	-1.54	1.01	6.0
gltD	-1.52	-1.42	0.93	6.0
ybeG	-1.52	-1.03	0.68	6.0
mtlD	-1.52	-1.92	1.26	6.0
yjdL	-1.52	-1.06	0.70	6.0
ybda	-1.51	-1.58	1.05	6.0
fhuA	-1.51	-1.59	1.05	6.0
feoB	-1.51	-2.04	1.35	6.0
hofD	-1.51	-1.22	0.81	6.0
fhuE	-1.50	-1.48	0.99	6.0
maeB	-1.50	-1.29	0.86	6.0
yidU	-1.49	-2.12	1.42	6.0
rplP	-1.48	-1.65	1.11	6.0
yiaO	-1.48	-1.06	0.72	6.0
yhaE	-1.48	-1.43	0.96	6.0
livH	-1.48	-1.02	0.69	6.0
rstB	-1.48	-1.34	0.91	6.0
yacH	-1.47	-1.11	0.75	6.0
yhfW	-1.47	-1.75	1.19	6.0
yjcX	-1.47	-1.84	1.25	6.0
yfaU	-1.47	-1.88	1.28	6.0
yaiX	-1.47	-1.28	0.87	6.0

Tab. 4.7 Genes identified by SAM as potentially significantly down-regulated when SecA830 was depleted in TH25 pTHS19. Data was derived from 2 independent replicates. d_i is the t-test score, r_i the average gene expression ratio ($\log_2$ transformed). The denominator of the t-test equation ($s_i + s_0$) is a measure of the error of the data.

4.3.3.2 *Translational analysis of gene expression*

Only a limited translational analysis was possible to complement the SecA830 depletion DNA-array experiment due to lack of appropriate antibodies. Nevertheless, the Western blot analysis showed that at the time samples for DNA-array were taken hardly any SecA was detectable in the cells (Fig. 4.78). The amount of DegP in the SecA-depleted cells was decreased compared to the amount of protein in the undepleted cells. This result confirmed the down-regulation of *htrA* (*degP*) identified by DNA-array. Unlike the Ffh- or FtsY depletion experiments, SecA830-depletion obviously did not lead to an increased transcription of heat shock genes. In addition, neither the amount of HslS nor GroEL was increased in the depleted cells.

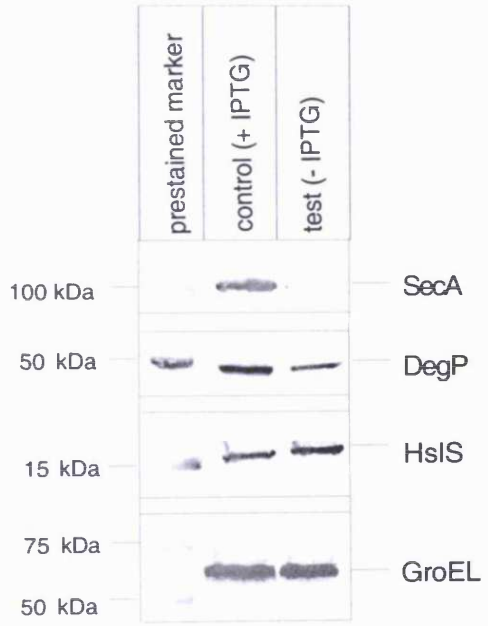


Fig. 4.79 Western blot analysis of selected proteins with the stated antibodies. An overnight culture of strain TH25 pTHS19 was grown in NZA medium with 100 µM IPTG and 5 µg/ml tetracycline. The cells were diluted 1/100 in NZA tetracycline medium with 100 µM IPTG (control) to induce SecA830 expression or without IPTG for depletion (test). Samples for 10% SDS-PAGE were taken after 3 hours of growth at an OD_{600} of about 0.52 (control) or after 5.5 hours at an OD_{600} of 0.18 (test).

4.3.4 Conclusion

In Ffh-depleted cells numerous genes encoding components involved in flagellar assembly were significantly down-regulated. This observation suggests either a direct connection between Ffh and flagellar gene expression or an indirect affect. As flagellar synthesis is an expensive process (McNabb, 1996) the cell might abolish flagellar assembly under severe stress to use energy for more vital prossesses.

Depletion of Ffh resulted in increased transcription of a subset of heat shock response genes. This effect was obviously stronger when FtsY was depleted instead of Ffh.

In contrast, no heat shock response genes were up-regulated in SecA830-depleted cells. Instead, the expression of the phage shock response operon *psp* was significantly increased. These results might indicate that cellular chaperones could play a role in targeting of inner membrane proteins.

5 Discussion

In this study a novel approach of *in vivo* inactivation of the essential Sec-machinery component SecA was further developed and improved. SecA was transformed into a target for site-specific proteolysis by the virus-derived TEV protease. To some extent this method was already successfully used in an earlier study (Mondigler and Ehrmann, 1996). Here, the cleavage efficiency was increased by preventing auto-regulated *de novo* synthesis of SecA and by cleavage of the unfolded nascent oligo-peptide chain in addition to the fully folded protein.

Via this system of target-specific proteolysis it was shown that inactivation of SecA not only affected the secretion of the periplasmic enzyme AP, but also the translocation of periplasmic domains of two inner membrane proteins. This result was also observed when SecA was depleted under the control of the *lac* promoter.

Our results support the notion of several other recent investigations (Qi and Bernstein, 1999; Schierle *et al.*, 2003) that SecA not only plays a central role in post-translational export, but is also involved in co-translational translocation of inner membrane proteins. Together with our observations here, it becomes more and more evident that the SecB/SecA pathway and the SRP/FtsY pathway are not strictly separate translocation routes but act either separately or in concert depending on sequence and structural properties of the exported protein.

5.1 Target-specific proteolysis of SecA via TEV protease

In previous studies on the role of SecA different approaches were used to inactivate its function. SecA activity has been blocked chemically by sodium azide or genetically via conditional lethal mutant alleles. Cabelli and coworkers used a SecA amber mutation to show that SecA was necessary for secretory protein translocation into *E. coli* membrane vesicles (Cabelli *et al.*, 1988). Amber mutations are mutations where one amino acid codon is replaced by the amber stop codon, which leads to a stop in translation. Such mutations can be suppressed by a temperature-sensitive amber suppressors at the

permissive temperature. At non-permissive temperatures the loss of the suppression leads to a stop in translation at the amber mutation site. A disadvantage of this method is that effects might manifest rather slowly, as several generations are necessary to deplete the target protein. In addition, growth at a non-permissive temperature might have other unwanted side-effects, for instance the induction of the heat shock response. This problem also arises when another type of conditional lethal mutation is used. A temperature-sensitive mutant of SecA was isolated that showed impaired protein secretion at 37°C and above (Oliver and Beckwith, 1981). This SecA allele has a single nucleotide exchange at position 43 (Schmidt *et al.*, 1988). Such single mutations are very much under selective pressure, thus, spontaneous formation of revertants that no longer show the temperature-sensitive phenotype are rather likely. In addition, it was shown that the SecA51(Ts) protein becomes trapped on the membrane at temperatures above 33°C (Cabelli *et al.*, 1991). This mutant protein might thus block inner membrane protein insertion simply by preventing the interaction of the ribosomes with the translocon.

In contrast to conditional lethal mutants an inhibition of SecA function with azide shows effects on protein translocation within minutes (Oliver *et al.*, 1990). However azide only partially inhibits SecA activity and has a negative effect not only on the translocation ATPase of SecA but also on other ATPases, e.g. the F_0F_1-ATPase. Therefore, the use of azide might lead to secondary effects.

Target-specific proteolysis of SecA as used in this study provided some advantages over the above mentioned methods of SecA-inactivation, e.g., cells were grown at a physiological temperature. TEV protease showed a high degree of sequence specificity (Dougherty *et al.*, 1989) and was regulated independently from the target protein. This allowed the induction of the protease at any time point during growth of the experimental culture. TEV protease could be expressed soluble in the cytoplasm or attached to the ribosome via the TF144 fragment without interfering with cell viability.

A search for homologues sequences of the heptapeptide cleavage sequence of TEV protease in *E. coli* showed no significant matches for the conserved positions (Fig. 5.1). Regions in two proteins matched the cleavage sequence in three out of four crucial positions. However, it is not likely that these sequences are recognised by TEV protease. In contrast, in *Bacillus subtilis* a 100% match for the hypothetical protein encoded by

gene *ywmA* was found. The function of this protein is unknown. Provided YwmA is not relevant for normal cell growth, TEV protease could be used as tool in *B. subtilis* as well. Apart from *E. coli*, TEV protease was expressed in *Saccharomyces cerevisiae* without negative effects on growth and viability (Smith and Kohorns, 1991).

E. coli		*B. subtilis*	
E-N-L-**Y**-F-**Q**-**S**	PCS	**E**-N-L-**Y**-F-**Q**-**S**	PCS
G-D-L-**Y**-F-**Q**-**S**	TldD	**E**-N-L-**Y**-F-**Q**-**S**	YwmA
E-N-L-**Y**-F-**Q**-**G**	PCS		
E-D-L-P-F-**Q**-**G**	PabB		

Fig. 5.1 Homology search by NCBI BLAST2 program (Altschul *et al.*, 1997) for TEV protease cleavage site (PCS) and bacterial proteomes. The essential 4 conserved amino acids of the PCS are shown in bold.

The expression of TEV protease in the cytoplasm did not always lead to a complete proteolysis of a SecA-PCS construct. Several important factors contribute to the successful cleavage of a target protein, for instance, the accessibility of the cleavage site, the position of the cleavage site in the protein, the localisation of the TEV protease in the cell as well as independent regulation of the expression of the target protein and the protease.

An interesting question was if proteolysis efficiency could be improved by cleavage of nascent polypeptide chains, as in this way the cleavage site was not hidden within the folded protein sequence. In cooperation with the diploma student Charlotte Conz the 27 kDa NaI-Pro fragment of TEV protease was fused to the first N–terminal 144 amino acids of trigger factor. TF144-TEV protease was bound to the large ribosomal subunit as efficiently as wild-type trigger factor (Conz, 2000). The binding site for trigger factor on the ribosome is located in proximity to the tunnel exit near ribosomal proteins L23 and

L29 on the back of the 50S subunit (Blaha *et al.*, 2003). Therefore, the protease is in close proximity to its target sequence during translation. The affinity of trigger factor for ribosomes is rather low and association as well as dissociation are slow processes (Maier *et al.*, 2003). Maier and coworkers also showed that the lifetime of the complex is of the order of seconds and enables trigger factor to remain bound during a major fraction of the time required for the synthesis of a protein. About half of the amount of trigger factor is found soluble in the cytoplasm (Hesterkamp and Bukau, 1996: Conz, 2000).

As the number of binding sites on the ribosome are the limiting factor for TF144-TEV protease association, the wild-type trigger factor was deleted in strains expressing this TEV protease hybrid protein. Charlotte Conz showed that under low salt conditions more TF144-TEV protease was bound to the ribosomes in a Δ*tig* strain than in the wild-type background (Conz, 2000). Although strain TH26 grew slightly faster than its Δ*tig*::*kan* derivative TH46, a growth difference between MC4100Δ*ara* and CC4 (MC4100Δ*ara* Δ*tig*::*kan*) was not observed (result not shown). Deuerling and coworkers also could not detect any defects in growth or protein folding in Δ*tig*::*kan* mutants (Deuerling *et al.*, 1999).

As shown by Western blot analysis the localisation of the TEV protease indeed affected the proteolysis efficiency (Fig. 4.34 and Fig. 4.35). Compared to GST-TEV protease the TF144-TEV protease could improve cleavage of SecA252 and SecA195, as more protein was proteolysed within a time period of 5 hours.

The same conclusion can be drawn from the observation that growth of TH47 (SecA195) was distinctly slowed down when TF144-TEV protease was induced, compared to growth of TH27 expressing the cytoplasmic GST-TEV protease (Fig. 4.39). It was expected that the same growth behaviour would be observed in strains TH26 and TH46 containing SecA252, when either cytoplasmic or ribosomal TEV protease were expressed. However, this was not the case (Fig. 4.38). Also, the Western blot analysis comparing proteolysis of SecA252 in these strains showed rather weak cleavage and no difference between the two TEV protease hybrid proteins (Fig. 4.37). This observation was unusual, as in former experiments SecA252 was often almost completely proteolysed and cannot be explained here. It is possible that a single mutation in the PCS at one of the weaker conserved positions lead to a decreased cleavage efficiency that could not be

increased by TF144-TEV. However, a sequencing reaction of the respective region would be necessary to clarify this question.

Taken together, proteolysis efficiency was increased by using TF144-TEV protease in several ways. First, association with the ribosome near the exit allows direct access to the cleavage site on the growing unfolded peptide chain during synthesis. Second, unproteolysed cleavage sites in the folded SecA protein are still a target of the un-associated TF144-TEV protease in the cytoplasm. A possibility to further increase the cleavage efficiency might be a direct fusion of TEV protease to the L29 protein at the ribosomal exit, provided that such a fusion would not interfere with normal protein synthesis.

Another aspect that influences the *in vivo* proteolysis system is the amount of target protein that is expressed in the cell. The possibility to regulate the amount of cleavable protein has the advantage that a low level of protein could be proteolysed faster and effects of this proteolysis event would become apparent earlier during cell growth.

The idea was to construct a strain in which the chromosomal wild-type *secA* is inactivated by a "knock-out" mutation and a *secA*-construct can be regulated by a tight promoter. The viability of such a strain is dependent on inducer supplementation. Such depletion strains were already existent for SecD, SecF and SecE (Pogliano and Beckwith, 1994; Traxler and Murphy, 1996) but not for SecA. To prevent overproduction of the SecA-construct from a multicopy plasmid, the *lac* promoter controlled construct was integrated into the chromosome as a single copy via the bacteriophage λInch. This method, developed by Boyd (Boyd *et al.*, 2000) provided a shuttle system to integrate all available SecA-PCS constructs successfully at the λ attachment site in strains that still contained the wild-type allele of *secA*. However, the appearance of S+ revertants that could grow lytically on the no longer λ immune colonies turned out to be a common problem at a late stage during the procedure. Several time-consuming steps of purification and dilution of the lysates were necessary to reduce the number of re-infections.

Western blot analysis showed that the chromosomal *secA*-constructs were expressed together with the wild-type allele of *secA* (Fig. 4.6). Therefore, the next step was to

eliminate the wild-type *secA*. A first approach was the insertion of a kanamycin resistance cassette into the *secA* sequence (results not shown). However, this method turned out to be unsatisfactory as strains regularly lost their kanamycin resistance, probably through recombination events between the two *secA* alleles. In a next attempt sequences adjacent to *secA* were PCR amplified with distinct restriction sites and cloned onto a vector. The idea was to insert an antibiotic resistance gene between the sequences at the position where normally *secA* would be located. Through recombination events between the homologues sequences on the plasmid and on the chromosome an exchange of the antibiotic resistance and chromosomal *secA* would occur. However, the cell did not tolerate plasmids containing the downstream regions of *secA*.

Finally, a new method to disrupt chromosomal genes, developed by Datsenko and Wanner (Datsenko and Wanner, 2000) proved to be successful. The λ Red recombinase system was used for recombination between regions adjacent of *secA* and a PCR product containing the antibiotic resistance and short homologues sequences to the target region. With this method wild-type *secA* was eliminated in all strains with *secA*-constructs inserted in the attachment site. However, during this procedure, all but one construct (SecA830) lost the ability of regulation via the *lac* promoter. One possible explanation for this phenomenon might be that a mutation in the *lac* promoter region leads to a loss of its function. However, loss of regulation occurred several times independently in cells containing different constructs, which makes this explanation less likely. Another possible explanation might be that the cell found a way to block or degrade the inducer IPTG to secure the constant expression of *secA* in the cell. SecA830 remained the only construct that was inducible with IPTG in a strain with eliminated wild-type *secA*. Proteolysis of this construct by TEV protease did not lead to a reduction in cell growth. Earlier studies have shown that the most distal 22 amino acids of SecA are responsible for SecB binding and that deletion of the carboxy-terminal 66-70 amino acids abolished SecA-SecB interaction, but did not affect the ability of SecA to export SecB-independent preproteins (Fekkes *et al.* 1997; Matsuyama *et al.*, 1990). As proteolysis of SecA830 was not absolutely complete by either TEV protease hybrid, a small amount of unproteolysed SecA830 might have been sufficient to facilitate export of SecB-dependent substrates.

Despite the *lac* promoter regulation defect, strains TH27 and TH47 (TH27Δ*tig*::*kan*) expressing TEV protease from either plasmid pTH9 or pTH10 were the first strains containing construct SecA195 that revealed a distinct growth defect upon SecA195 proteolysis. When SecA195 was proteolysed in strain MM63 or CC2 (MM63Δ*tig*::*kan*) no differences in the growth behaviour in the induced or uninduced cultures were observed (results not shown) although the number of surviving cells measured by viability spot tests was drastically reduced (Conz, 2000).

One of the differences between MM63 and TH27 that might be responsible for the obviously stronger proteolysis effect is the loss of the autoregulation of *secA* expression in strain TH27. Strain MM63 (Mondigler, 1997) was constructed via a recombination event between a plasmid-encoded *secA*195 and the temperature-sensitive *secA* allele *secA*51(Ts) (Schmidt *et al.*, 1988) in strain MM52. Although this is an elegant one-step method to insert a *secA*-construct in the chromosome there are some disadvantages. One disadvantage is that the construct remains at its natural position. Consequently it is still under wild-type promoter control and underlies autoregulation on a translational level because the region between the upstream gene*X* (*secM*) and the *secA*-construct is still present. Therefore, if the amount of SecA in the cell is reduced, SecA dissociates from its binding site on the gene*X*-*secA* mRNA and enables *de novo* synthesis of SecA. In addition, *secA*-constructs with more than one inserted cleavage site or cleavage sites too far away from the 5'end of *secA* could not be exchanged via recombination.

An important factor in the successful proteolysis of SecA was the position of the TEV protease cleavage site within the *secA* sequence. An optimal position is characterised by several features. The insertion of the cleavage site must not interfere with normal SecA synthesis, folding, stability of the protein and function. In addition, cleavage at this site should lead to total inactivation of the functional protein. It was suggested, that cleavage of SecA252 in strain MM58, although complete, did not produce a secretion defect because the proteolysed domains probably remained associated by alternative bonds (Mondigler, 1997).

To find an optimal position for the PCS more than 20 different sites within the *secA* sequence were chosen. Cloning of most constructs using vector pEDIE-2 was performed

by the company GATC AG (Konstanz, Germany). However, just seven constructs could be successfully cloned (SecA60, SecA295, SecA471, SecA497 by GATC AG; SecA229, SecA296 and SecA518 in this study). Complementation tests showed that from all constructs just four (SecA229, SecA295, SecA296 and SecA518) were able to restore growth at 42°C in a strain containing the temperature sensitive *secA*51(Ts) (Fig. 4.22). However, colonies grew not as fast as when the temperature sensitive phenotype was restored with wild-type SecA from plasmid pMF8. A Western blot analysis showed that all four constructs on pEDIE-2 derived plasmids were not stably expressed (Fig. 4.20). Probably a small amount of functional SecA-PCS that might not be detectable on a Western blot was sufficient to facilitate growth of the cells to a certain extent. Alternatively, probably the SecA fragments remained functional. Expression in different protease minus strains and elimination of the insignificant 12 amino acids upstream of the PCS that might have contributed to recognition by cellular proteases did not improve the stability of the SecA-PCS proteins. From the seven SecA-constructs only SecA60 was stably expressed but could not fulfil normal SecA function, probably because the presence of the PCS at the N-terminus of the protein prevented membrane insertion of SecA during the translocation process (Conz, 2000).

The positions for introducing a PCS were chosen on the basis of structural analysis of SecA from *Bacillus subtilis* (Hunt *et al.*, 2002; J. Hunt pers. communication). These regions were predicted to be surface exposed providing accessibility to the TEV protease and minimising interference with protein folding by the inserted 22 amino acids.

The sequence homology between *B. subtilis* SecA and *E. coli* SecA is about 50% (alignment not shown) which indicates that the folded proteins might take up a similar conformation. The alignment of the SecA-protein sequence of the three species *E. coli*, *B. subtilis* and *Mycobacterium tuberculosis* showed that the PCS in most SecA-constructs was located in poorly conserved regions (Fig. 5.2). For example, such regions might be insertions that are most likely located at the surface of the protein, like the regions around amino acids 252, 295, 296 and 518 of the *E. coli* SecA. Weakly conserved regions that differ not only in their amino acid sequence but also in their secondary structure, like the region around residue 830 (Fig. 5.3), also provide suitable positions for the insertion of a PCS as they are not likely to contain important functional domains.

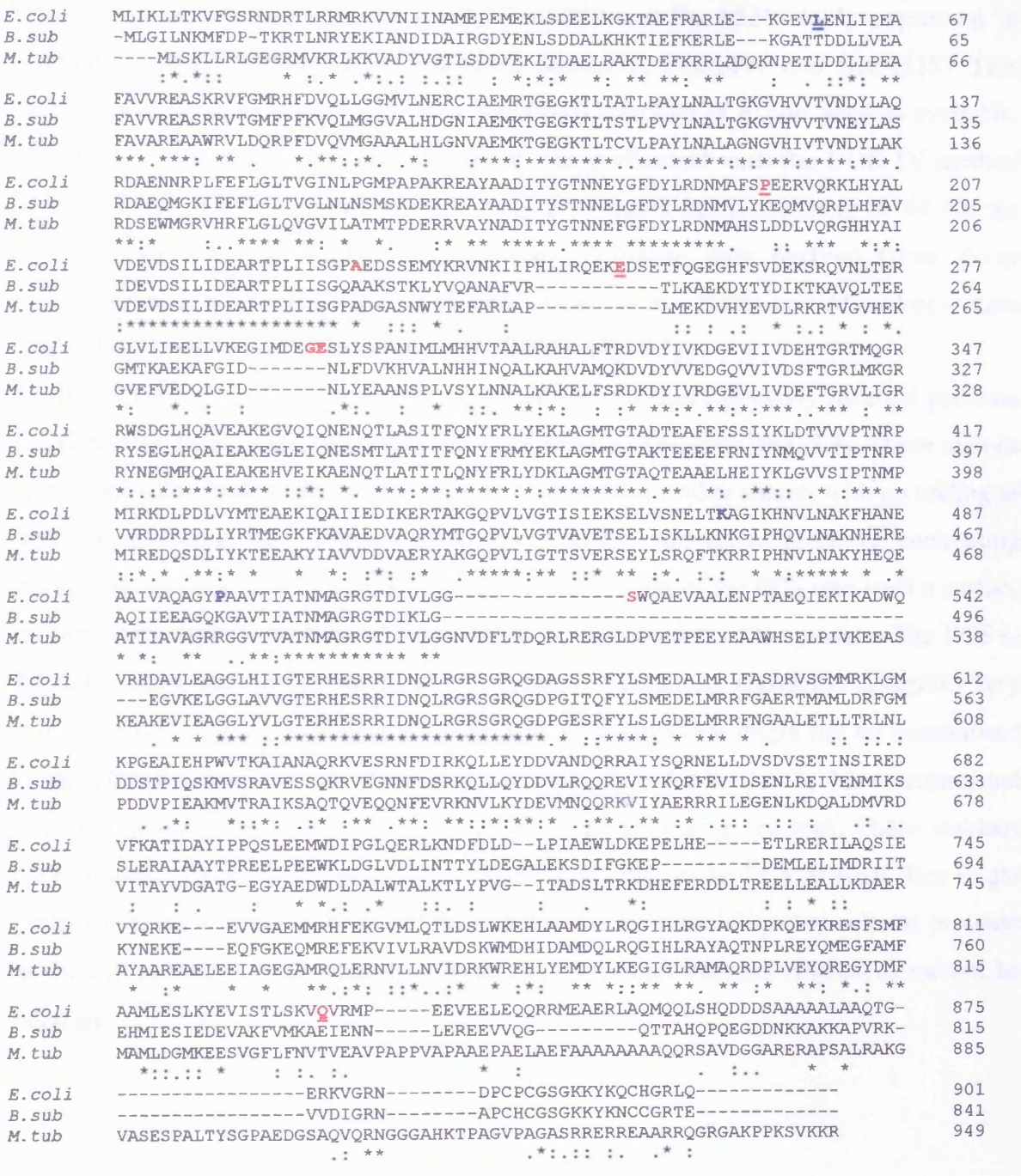


Fig. 5.2 Alignment of SecA-protein sequences from *E. coli*, *B. subtilis* and *M. tuberculosis* by CLUSTAL W (Thompson *et al.*, 1994). 33.88% of the amino acid residues are identical in the 3 species (*). 16.94% of the residues are strongly similar (:) and 8.32% are weakly similar (.). Marked are amino acids behind which a PCS was cloned either in this study or in previous studies (Bolek, 1996; Mondigler 1997; Conz, 2000). Residues marked in red show a SecA-construct that did complement SecA51(Ts) in contrast to blue residues. Underlined residues show SecA-constructs that were stably expressed.

Why are constructs SecA60, SecA195, SecA252 and SecA830 stably expressed in contrast to SecA229, SecA295, SecA296, SecA471, SecA497 and SecA518? This question is difficult to answer as to date no crystal structure of *E. coli* SecA is available. Therefore, a secondary structure prediction was performed with the GOR IV method (Garnier *et al.*, 1996). As this method only provides a mean accuracy of 64.4% the structure was compared also to secondary structure data derived from X-ray crystallography of *Bacillus subtilis* SecA (Hunt *et al.*, 2002) and *Mycobacterium tuberculosis* SecA1 (SEA1) (Sharma *et al.*, 2003) (Fig. 5.3).

Analysis of the secondary structures of SecA showed that the newly inserted protease cleavage sites were located in most cases in random coil regions (Fig. 5.3). These regions take up irregular configurations that may include bends or other shapes with no coiling as well. More sharply bent regions often occur at the surface of proteins, connecting domains located more deeply in the protein. The insertion of the PCS into such a surface exposed loop might contribute to a destabilised configuration of the protein. The PCS in the stably expressed SecA-constructs was located in α-helical segments, in regions very close to α-helical segments or in very short random coils that might not be destabilised by the additional amino acids. Although this is also true for SecA518, this construct and other unstable constructs are located near glycine and proline residues. These residues might indicate bends in the protein structure. Insertion of the PCS at such sites might contribute to an unstable configuration. In addition, the possibility that cellular protease might still recognise and proteolyse even the shortened inserted sequences cannot be ruled out.

Fig. 5.3 Details of the secondary structure of the SecA protein in *E. coli*, *B. subtilis* and *M. tuberculosis* 1) predicted by GOR IV (Garnier *et al.*, 1996) and 2) derived from X-ray crystallography (Hunt *et al.*, 2002; Sharma *et al.*, 2003; data available from the protein data bank). Residues behind which a PCS was cloned are marked. Abbreviations: c = random coil, e = beta bridge, h = α-helix, t = turn.

Taken together, the insertion of PCS at numerous positions in the *secA* sequence turned out to be difficult. This is not unexpected as the insertion of a PCS is equivalent to the insertion of a mutation into the sequence. As SecA is an essential component for cell viability and interacts with numerous partners, the insertion of a PCS is not tolerated at a random position. In contrast, the insertion of several PCS in the sequence of the outer membrane protein TolC for topology studies has been successful (Mondigler, 1997). TolC insertions were obtained using a transposon, while the positions of the SecA inserts were chosen based on plausible considerations considering the crystal structure of *B. subtilis* SecA. Given the results with SecA and TolC insertions, the randomised approach with a transposon seems to be the better choice, perhaps because the number of inserts that can be analysed is larger. Also, the surprising cloning problems observed with SecA would be avoided when using a transposon.

5.2 Insertion of inner membrane proteins is dependent on Ffh, FtsY and SecA

The role of Sec-components, especially SecA, in the SRP-dependent translocation pathway is still controversial. It was suggested that the signal recognition particle and its receptor are necessary and sufficient to target and integrate membrane proteins into the inner membrane (MacFarlane and Müller, 1995; de Gier *et al.*, 1996; Ulbrandt *et al.*, 1997). Consequently the SecA/SecB mediated pathway and the SRP/FtsY targeting route would operate as two distinct targeting and translocation systems (Koch *et al.*, 1999). This suggestion was strengthened by the findings that MalF integrated normally into the inner membrane in cells with impaired SecA activity (McGovern and Beckwith, 1991). Moreover, it was shown that SecA was dispensable for the insertion of the inner membrane proteins FtsQ (Scotti *et al.*, 1999), MtlA (Werner *et al.*, 1992) and ProW (Whitley *et al.*, 1994).

However, on the other hand evidence was found that SRP as well as SecA were engaged for the insertion of a subset of inner membrane proteins. In contrast to what was found by McGovern and Beckwith, studies from the same group demonstrated a dependency on SecA for proper MalF insertion (Traxler and Murphy, 1996). This result was in accordance with the results of Jander and coworkers, who found that inhibition of SecA with sodium azide lead to the biotinylation of two MalF-PSBT fusions within the second periplasmic loop (Jander *et al.*, 1996).

In addition, it was shown that the assembly of several other inner membrane proteins, for instance Lep (Wolfe *et al.*, 1985), SecY-AP and AcrB-AP fusion proteins (Akiyama and Ito, 1989; Qi and Bernstein, 1999) and YidC (Urbanus *et al.*, 2002; Koch *et al.*, 2002) was dependent on SecA.

These contradicting observations might be due to insufficiently sensitive assay systems or incomplete inactivation of SecA activity. It cannot be ruled out, that some or all inner membrane proteins have a high affinity for Sec components. Thus a strong inhibition of SecA activity might be necessary to observe effects on membrane protein insertion.

Here, we used a SecA-depletion strain that showed a growth defect when SecA expression was not induced and a strain in which SecA could be efficiently proteolysed

by TEV protease. In addition, to detect defects in membrane protein insertion we used a very sensitive assay where a biotinylatable domain was fused to exported domains of membrane proteins as well as to a secreted protein (Jander *et al.*, 1996). With this method a detection of deficiencies in the export of these domains or proteins is possible.

Biotinylation of the AP-, MalF274- and FtsQ-PSBT fusion proteins occurred upon inactivation of SecA195 in strains TH27 and TH47 via TF144-TEV protease or cytoplasmic TEV protease, and upon depletion of SecA830 in strain TH25 pTHS19. The biotinylation effect was slightly weaker when SecA195 was proteolysed by soluble TEV protease. Inactivation of SecA195 by ribosome-attached TEV protease and depletion of SecA830 resulted in similar intense biotinylation bands. Very weak biotinylated bands of FtsQ-PSBT and stronger biotinylated bands of AP-PSBT and MalF-PSBT were obtained upon induction of these proteins with 0.2% arabinose even under non-depletion or non-inactivation conditions. This observation might suggest an inhibitory effect of the PSBT domain on secretion and/or insertion of proteins. However, this is not likely, as when the amount of AP-PSBT and MalF-PSBT was reduced, no biotinylated band was visible when SecA was not depleted or proteolysed. In addition, other studies have shown that the PSBT *per se* has no negative effect on secretion or insertion (Jander *et al.*, 1996; Reed and Cronan, 1991). More likely, this background biotinylation was due to the high amount of fusion protein expressed under the control of P_{araBAD} that probably overloaded the secretion and insertion machinery. The *araBAD* promoter was not tightly regulated in these experiments. However, this was a positive side-effect here, as it allowed the reduction of the protein amount. A better solution for future experiments would be to reduce the plasmid copy number by exchanging the pBR322 origin of replication against a low copy origin.

AP is a secretory protein. Therefore, biotinylation of the AP-PSBT protein was expected when SecA function was impaired. More unexpected was the result that MalF274-PSBT showed the same biotinylation patterns as AP-PSBT under SecA-inactivation. We showed here that at least a part of MalF needed SecA for proper membrane insertion. This result is in accordance with the observation of Jander and coworkers, that two MalF-PSBT fusions were biotinylated when SecA activity was blocked by sodium azide (Jander *et al.*, 1996).

We also showed that the insertion of the small membrane protein FtsQ into the inner membrane was dependent on SecA. However, the extent of the SecA-dependence of FtsQ was not altogether clear. As no FtsQ-antibody was available, it was not possible to determine the amount of PSBT-fusion protein upon induction with arabinose or whether a residual amount of protein was synthesised without induction. No biotinylation effect was detectable without arabinose induction. This could either mean that the regulation of the *araBAD* promoter was tight in this case, or that FtsQ is not as dependent on SecA as AP and MalF274 because a higher amount of protein was necessary to produce a biotinylation effect. On the other hand, provided the amount of fusion protein was the same in all three cases, the observation that induction of FtsQ was necessary for an effect could mean that FtsQ is a better substrate for SecA than the other two proteins. However, as the biotinylation effect with cytoplasmic TEV protease was as strong as with ribosomal attached TEV-protease it is more likely that the dependency of FtsQ on SecA is less strong. In a previous study it was shown that SecA was not required for the initial insertion of FtsQ into the membrane in an *in vitro* system (Scotti *et al.*, 1999). However, these observations do not contradict our results here, as SecA may well be involved in FtsQ-insertion at a later stage, especially in the translocation of the C-terminal periplasmic domain. In addition, SecA was found in close proximity to nascent FtsQ in a cross-linking experiment (Valent *et al.*, 1998).

Both membrane proteins tested here contain extended periplasmic domains or loops that have to be translocated across the inner membrane. Although a requirement for SecA has also been shown for other inner membrane proteins with large periplasmic domains like leader peptidase (Wolfe *et al.*, 1985) and YidC (Koch *et al.*, 2002) the translocation of large periplasmic domains is not always SecA-dependent (Fröderberg et al., 2003). The authors showed SecA dependency of a ProW derivative containing the large (100 amino acids) N-tail and the first transmembrane domain of ProW followed by the P2 (240 amino acids) domain of leader peptidase in the cytoplasm. However, the insertion of a similar model protein containing three lysines in the cytoplasm instead of the P2 domain was not dependent on SecA.

The MalF274-PSBT and FtsQ-PSBT proteins were biotinylated upon depletion of either Ffh or FtsY. This effect was even detectable when the fusion proteins were not induced

with IPTG. As observed with the *araBAD* promoter also the *tac* promoter was leaky, so that a small amount of fusion protein was made even without induction.

Our results are in accordance with the findings of Tian and Beckwith, that the MalF-PSBT periplasmic fusion J and FtsQ-PSBT were biotinylated in *ffs*, *ffh* and *ftsY* mutant strains (Tian and Beckwith, 2002). They also confirm the suggested function of Ffh and FtsY in the insertion of inner membrane proteins (de Gier *et al.*, 1996; Seluanov and Bibi, 1997; Ulbrandt *et al.*, 1997).

Biotinylation of the periplasmic enzyme alkaline phosphatase (AP) was observed under Ffh- or FtsY-depletion when synthesis of AP-PSBT was induced with 100 µM IPTG. Part of the biotinylation signal might be due to the high amount of fusion protein, as even without depletion AP-PSBT was partly biotinylated. Here, the same explanation is likely as in the SecA-inactivation experiments, that overproduction of the fusion protein might lead to a slowed translocation activity. A negative effect of IPTG on the system is less likely, as no such effect was observed in similar assays (Tian *et al.*, 2000, Tian and Beckwith, 2002).

Nevertheless, the clearly stronger signal under depletion conditions indicated that the secretory protein AP might be dependent on Ffh and FtsY for membrane targeting. When AP-PSBT was induced with less IPTG (10 µM) a biotinylation signal was only observed under FtsY-depletion but not Ffh-depletion. This observation might indicate a more important role of FtsY in targeting of AP. On the other hand, depletion of FtsY has been shown to inhibit SecY expression in contrast to Ffh-depletion (Herskovits and Bibi, 2000). However, this effect was visible in their experiment only after a longer depletion time than was used here and might not be the reason for the differences in biotinylation of AP-PSBT.

An effect of Ffh- or SRP-depletion on AP translocation has been observed in other studies as well. Phillips and Silhavy observed an export defect for some periplasmic proteins in Ffh-depleted cells (Phillips and Silhavy, 1992). Among these proteins were AP, β-lactamase and ribose-binding protein (RBP). The depletion of SRP but not trigger factor also led to an accumulation of AP and AP-signal sequence mutants (Kim *et al.*, 2001). As translocation of AP, β-lactamase and RBP is considered to be SecB-independent (Collier *et al.*, 1990; Kumamoto and Beckwith, 1983) it has been speculated

that SRP might replace SecB during export of these proteins (Phillips and Silhavy, 1992). Recently it was shown that this speculation is not true, at least concerning β-lactamase, as export of this enzyme was demonstrated to be not dependent on SRP (Beha *et al.*, 2003). In general, the problem of secondary effects caused by depletion of Ffh and FtsY cannot be totally ruled out, as the membrane components of the Sec machinery are probably dependent on Ffh and FtsY for targeting and insertion themselves. Therefore, depletion of Ffh or FtsY might reduce the concentration of functional Sec-translocons and indirectly affect the export of otherwise SRP/FtsY- independent substrates.

Taken together, the biotinylation assays carried out in this study strengthen the notion that some inner membrane proteins require the co-operative action of the SRP/FtsY pathway and SecA for proper insertion. The targeting of such proteins to the membrane thus probably occurs co-translationally via SRP and FtsY, whereas the subsequent insertion and /or translocation of the periplasmic domain is dependent on SecA activity. In eukaryotic cells a tight seal between the ribosome and the translocon is formed after the ribosome-nascent chain complex is targeted to the ER membrane (Crowley *et al.*, 1993). The polypeptide is then pushed through the channel via continued elongation of the peptide chain. An equivalent of SecA is not necessary to provide additional translocation energy. In contrast, in bacteria SecA generates energy for the post-translational export of preproteins. Probably in prokaryotic cells a tight seal between the translating ribosome and the translocon is not possible, or additional energy is necessary for co-translational translocation. It still remains unclear how the ribosome, the nascent chain and SecA interact at the translocation site. Zito and Oliver recently found that SecA possesses a ribosome-translocon dissociation activity (Zito and Oliver, 2003). Their results suggested that SecA releases translocon-bound ribosome during the translocation process. The ability of SecA to interact with the ribosome might not only be important in the Sec-dependent secretion mechanism but also during translocation of otherwise co-translationally exported domains or proteins. Alternatively, SecA might be required in the SRP/FtsY pathway not merely to provide energy for the translocation of large periplasmic loops, but to clear the translocon of unproductive ribosomes to facilitate

binding of the ribosome-nascent chain complex. However, this possibility would mean that probably all SRP/FtsY substrates require SecA.

The extent of the SecA-dependency for proper assembly seems to differ considerably between the various membrane proteins. This was indicated here, as the effect of SecA-inactivation on FtsQ insertion appeared to be slightly weaker than on the insertion of MalF274, although both proteins contain large periplasmic domains. Our results are in accordance with recent studies that revealed more versatile and flexible mechanisms of membrane protein biogenesis and protein translocation than previously thought (Fröderberg *et al.*, 2003; Neumann-Haefelin *et al.*, 2000).

For instance, the translocation of the autotransporter protein hemoglobin protease, which is secreted by a human pathogenic *E. coli* strain, has been shown to be dependent on both SRP and SecA (Sijbrandi *et al.*, 2003). A role of SecA in co-translational export was also very recently demonstrated by Schierle and coworkers (Schierle *et al.*, 2003). The authors showed that the signal sequence of the periplasmic protein DsbA directed the cytoplasmic protein thioredoxin as well as the periplasmic protein MBP into the SRP-pathway. They also showed that export of thioredoxin with DsbA signal sequence and DsbA itself was dependent on SecA.

5.3 Changes in gene expression profiles

The availability of the complete genome sequence of *E. coli* and recent progress in DNA array technology have made it possible to investigate changes in global gene expression under various experimental conditions or as a result of different genotypes of the cells. However, the statistical methods to analyse a large data set and to assess significant features are still under discussion. Common methods range from applying simple fold-change thresholds to performing t-tests and more complex statistical test (Arfin *et al.*, 2000; Barbosa and Levy, 2000; Long *et al.*, 2001).

The data sets obtained by DNA array experiments in this study were analysed by SAM, a statistical method for analysing microarray data recently proposed by Tusher and coworkers (Tusher *et al.*, 2001). This method detects significant differential gene expression by assigning a t-test score for each gene on the basis of its change in gene expression relative to the standard deviation of repeated measurements. Genes with scores greater than an adjustable threshold are deemed potentially significant. SAM uses permutations of the data sets to identify the percentage of genes called significant by chance. This percentage is the false discovery rate (FDR), calculated for each chosen threshold. The authors provided evidence that SAM successfully identified induced genes in an artificial data set and estimated the FDR with reasonable accuracy. Significant genes are listed by their t-test score. Calculation of absolute transcript levels of genes or comparing fold change values between different genes might not be sensible because this would require equal efficiency of reverse transcriptase reactions and of hybridisation (Richmond *et al.*, 1999).

To gain statistically reliable results 4 to 5 independent replicates were produced for every DNA array experiment, with one exception. For the SecA-depletion experiment only 2 independent array measurements could be performed due to lack of time and material. A number of experimental factors could have affected the reproducibility and accuracy of the replicates. The main source of experimental error arose from differences among RNA preparations. A successful method to reduce variances between RNA samples was the use of RNAprotect Bacteria Reagent (QIAGEN). This reagent stabilises RNA prior to isolation by preventing degradation or gene induction during handling.

Other factors during the experimental procedure that might have led to artefacts in the gene-expression analysis were problems like cross-hybridisation, PCR failures, misapplied DNA spots on the membrane or uneven distribution of the radioactive sample during hybridisation.

The number of replicates, visual comparison of the scanned images of the DNA array membranes and the analysis of the data with two different statistical methods helped to keep the experimental error as minimal as possible.

Discrepancies between the results obtained through the array experiments on the transcriptional level and Western blot analysis on the translational level may have arose for various reasons. For instance, Western blot analysis showed an increased protein amount of HtpG and GroEL in Ffh- and FtsY-depleted cells. However, DNA array analysis showed an up-regulation of gene-expression of the two genes only in FtsY-depleted cells. Probably the expression ratios for these genes differed too much between replicates to be called significant or the up-regulation in Ffh-depleted cells occurred only on the protein level.

Translational regulation or rather stable mRNAs could also be the reason for the observation that Ffh, FtsY and SecA830 were hardly detected on Western blots after a certain time of depletion, but array analysis showed no statistically significant down-regulation of the corresponding genes.

The most obvious functional group of genes that was affected by Ffh- or FtsY-depletion was the heat shock response group. Under both depletion conditions several genes encoding heat shock proteins were significantly up-regulated. This effect was more pronounced in FtsY-depleted cells than in Ffh-depleted cells. In addition, increased levels of heat shock proteins like DnaK, HtpG, GroEL and HslT/HslS could be detected on Western blots. Our results are in accordance with the finding that also moderate SRP deficiencies increased the levels of DnaK and GroEL (Bernstein and Hyndman, 2001).

The up-regulation of the heat shock genes upon depletion of either Ffh or FtsY might have more than one reason. One explanation could be a general response to a stress condition where mislocalised or misfolded proteins destined for the inner membrane have to be degraded or held in an export-competent state. Bernstein and Hyndman found that

under reduced levels of SRP the heat shock proteases Lon and ClpQ became essential for viability (Bernstein and Hyndman, 2001). The authors concluded that SRP might be essential because efficient targeting prevents the toxic accumulation of aggregated proteins in the cytoplasm. An increase in components that help to degrade mislocalised proteins in SRP-deficient cells might therefore be important for cell viability. Here we found that in FtsY-depleted cells the expression of several heat shock proteases like HslV and HtpX was induced. It cannot be ruled out however, that proteases become important because an unknown function of Ffh or FtsY is disrupted.

The *in vivo* function of the two small heat shock proteins HslT (IbpA) and HslS (IbpB) is still not clearly defined. They were shown to be dispensable at high temperatures (Thomas and Baneyx, 1998) but overexpression resulted in resistance to heat and oxidative stress (Kitagawa *et al.*, 2000), and they were found to bind to endogenous *E. coli* proteins that aggregated in the cell after heat shock (Laskowska *et al.*, 1996). It was shown that HslS acted as a large oligomer and prevented irreversible aggregation of model proteins *in vitro* (Shearstone and Baneyx, 1999). Only recently it was demonstrated that the small heat shock proteins form a functional triade with ClpB and the DnaK system to reverse protein aggregation *in vitro* and *in vivo* (Mogk *et al.*, 2003).

DnaK, a member of the Hsp70 family, belongs to one of the two major chaperone systems of the cell. This chaperone is involved in folding of nascent chains (Teter *et al.*, 1999), refolding of proteins after stress or heat damage, general proteolysis of proteins, phage λ replication and regulation of the heat shock response (Gross, 1996). It has also been suggested that DnaK plays a role in protein targeting and it was shown that it could substitute for SecB function and facilitate export of certain proteins (Wild *et al.*, 1992; Qi *et al.*, 2002). A role in membrane targeting of secretory or inner membrane proteins has also been discussed for the second major chaperone GroEL and the Hsp90 homolog HtpG (Shirai *et al.*, 1996; Bochkareva *et al.*, 1996). In addition, GroEL was also shown to interact with SecA *in vitro* and stimulate its release from the membrane (Bochkareva *et al.*, 1996). Therefore, another possible explanation for the increased level of heat shock proteins in Ffh- and FtsY- depleted cells, particularly of chaperones, is that they might provide alternative targeting routes to the membrane.

DnaK was shown to interact not only with unfolded proteins but also with detergent-solubilised membrane proteins on account of their hydrophobic sequences (de Crouy-Chanel *et al.*, 1999). In the absence of either Ffh or FtsY the translation of the inner membrane protein proceeds. DnaK, GroEL and the small heat shock proteins might prevent folding of the newly synthesised protein or shield the hydrophobic regions from the hydrophilic environment. Although GroEL seems to interact with SecA (Bochkareva *et al.*, 1998), the question of how the chaperone-protein-complex (probably still in conjunction with the ribosome) is received on the membrane remains to be solved.

If it were the case that heat shock proteins complemented the targeting function of Ffh or FtsY, loss of viability by depletion of these components might result from essential alternative functions of these proteins. It was observed that depletion of Ffh lead to a defect in cell division, evident through an elongated cell phenotype (Phillips and Silhavy, 1992). The DNA array experiments carried out here showed that cell division genes *ftsN* or *ftsH* were negatively affected by depletion of Ffh or FtsY respectively. In addition, cells depleted of FtsY showed up-regulation of several genes that encode components for the uptake and metabolism of amino sugars. These sugars are necessary to form constituents of the bacterial cell wall. For instance, the mRNA levels of the sialic acid transporter NanT that takes up the amino sugar N-acetylneuraminic acid and its homolog YjhB were increased. Gene expression of *nanA* was also up-regulated. The aldolase NanA helps to convert the NanT substrate into a cell wall precursor (Plumbridge and Vimr, 1999). The up-regulation of these genes might indicate a possible involvement of FtsY in cell wall assembly.

In Ffh-depleted cells the expression of several genes that are involved in flagellar assembly were significantly down-regulated. The regulation of the flagellar biogenesis is a complex interwoven system on transcriptional and translational levels that is not yet completely understood. The operons of the flagellar regulon are organised in a hierarchy of three classes: early, middle and late genes (for review see Chilcott and Hughes, 2000). The early operon encodes the transcriptional activators FlhC and FlhD that regulate the expression of the middle and late genes. Among the signals that affect the transcription of these regulators are cAMP-CPR (Yokota and Gots, 1970), growth phase (Nishimura and

Hirota, 1989) and cell cycle control (Mizushima *et al.*, 1994; Prüss and Matsumara, 1996). The middle genes encode structural and assembly proteins for the biogenesis of the flagellar hook-basal body. From these middle genes *flgB* and *flgEFG*, all included in one operon, were down-regulated upon Ffh-depletion. Moreover, also expression of the middle gene *fliA* was negatively affected. The gene product of *fliA*, σ^{28} plays a central role in flagellar gene regulation. The alternative sigma factor is specific for promoters of the late genes, but as recently shown, might also activate gene expression of the master operon *flhCD* (Kutsukake, 1997). Consequently, the number of flagella is dependent on the cell cycle and can be increased by either increased levels of FlhCD or σ^{28} activity. Array analysis revealed that also two late genes, *fliC* and *tsr*, were down-regulated in Ffh-deficient cells. This is most likely due to the reduced level of σ^{28}.

A second important middle gene is the negative regulator FlgM that acts as an anti-sigma factor on σ^{28}, preventing transcription of the late genes. FlgM is secreted through the flagellum-specific type III pathway. Thus, by decreasing concentration of this protein the sigma factor σ^{28} is de-repressed. In this elegant way the FlgM protein senses the developmental state of flagellar assembly, as a completed hook-basal body is required for secretion. In a next step late genes encoding components of the external filament as well as chemotaxis and motility components are induced. How Ffh might be involved in the regulatory network of flagellar biogenesis is not at all clear.

In interpreting the above results it is important to note that by using depletion strains to study an essential cellular process secondary effects might obscure the true function of the depleted gene product. This might be due to the prolonged growth period that is necessary to exhaustively deplete a gene product. A functional analysis of SRP by investigating a temperature-sensitive *ffh* mutant lead to the conclusion that Ffh has no other function than in the targeting of inner membrane proteins (Park *et al.*, 2002).

In this light, at least some of the effects of Ffh- or FtsY-depletion on gene-expression observed here are likely to be of secondary nature. The biogenesis of flagella is a costly process (McNabb, 1996), thus, reducing the expression of flagellar genes might be beneficial (Edwards *et al.*, 2002). Probably also heat shock genes play a role in the reduction of the flagellar gene expression. It was shown that DnaK and DnaJ were required for motility (Shi *et al.*, 1992). Availability of these proteins for induction of

flagellar synthesis might be reduced in Ffh- and FtsY- depleted cells, as the heat shock proteins are engaged in rescuing newly synthesised inner membrane proteins.
The reduced expression of several genes encoding ribosomal proteins in cells deficient in either Ffh, FtsY or SecA830 might be linked to the fact that under sub-optimal growth conditions the number of ribosomes is decreased (Keener and Nomura, 1996; Tao *et al.*, 1999).

Depletion of SecA830 had a large effect on gene expression. Among the affected genes were numerous genes encoding inner membrane proteins responsible for export, transport, signal transduction and other tasks. Obviously much more genes were negatively affected than positively. The phage shock operon *pspABCDE* was the most noticeable functional group that showed increased gene-expression. The regulation of this σ^{54} dependent operon is rather complex and is mediated via protein-protein interactions (Adams *et al.*, 2003). The membrane-associated and cytoplasmic PspA protein is a negative regulator of the *psp* operon (Weiner *et al.*, 1991) without binding to DNA (Dworkin *et al.*, 2000). The proteins PspB and PspC are both anchored in the inner membrane and act co-operatively as positive regulators of the *psp* operon (Weiner *et al.*, 1995). Interestingly, the requirement for the various regulatory proteins is dependent on the induction signal (Model *et al.*, 1997). For instance, PspB and PspC are required for induction of the phage shock operon upon filamentous-phage infection but only partially required upon osmotic shock (Weiner *et al.*, 1991). In contrast, heat shock induction is totally independent on these two activator proteins (Weiner *et al.*, 1991). In addition to the stimuli mentioned above it has been demonstrated that a number of other various stress conditions induced the expression of the *psp* operon. The *psp* genes were induced by proton ionophores (Weiner and Model, 1994), disruption of fatty acid biosynthesis (Bergler *et al.*, 1994) and exposure to organic solvents (Kobayashi *et al.*, 1998). The PspA protein is a major component of the limited protein synthesis that occurs in late stationary phase (Weiner and Model, 1994). Expression of PspA was also induced upon YidC-depletion (van der Laan *et al.*, 2003). YidC is involved in the biogenesis of inner membrane proteins and its depletion affected the functional assembly of the cytochrome *o* oxidase and the F_1F_0 ATPase complex. Thus, the proton motive force in these cells was

reduced. In accordance with our results it was shown that PspA synthesis was induced in *secA*, *secD*, and *secF* mutants but not in *secY* and *secE* mutants (Kleerebezem and Tommassen, 1993). In addition, the expression of a mutant form of the outer membrane protein PhoE that blocked the secretion apparatus resulted in PspA induction (Kleerebezem and Tommassen, 1993). The authors suggested that the inducing signal of PspA induction was the accumulation of precursor proteins within the export machinery in the inner membrane. Later it was shown that expression of a PhoE mutant protein in a *psp* mutant strain resulted in a decreased proton motive force (Kleerebezem *et al.*, 1996). It was therefore suggested that PspA maintains the PMF under conditions when protons might leak through an obstructed Sec channel. However, details of PspA function still remain unclear. A common feature of all stimuli that induce phage shock response is that they probably all injure the inner membrane integrity. Therefore, rather than playing a direct role in translocation, PspA might be involved in maintaining the membrane integrity (Adams *et al.*, 2003).

Regarding protein translocation, another interesting gene that was up-regulated under SecA-depletion is *msyB*. The function of the highly acidic *mysB* gene product is largely unknown. However, the gene was discovered in a multicopy suppression experiment where MsyB enhanced export activities in a *secY24* mutant strain (Ueguchi and Ito, 1992). Characterisation of *msyB* by these authors showed that the gene is part of an operon, together with a gene encoding a multipath integral inner membrane protein. It was speculated that MsyB might interact with the cytoplasmic domain of this membrane protein and probably in a similar manner also with SecY (Ueguchi and Ito, 1992). Why SecY24 and depletion of SecA830 both affected *msyB* expression remains to be resolved as MsyB did not improve export in a *secA*51(Ts) mutant (Ueguchi and Ito, 1992).

Surprisingly, in contrast to Ffh- and FtsY-depleted cells, no heat shock induction was observed upon SecA-depletion. This observation probably might be due to the prolonged growth period that was necessary to extensively deplete SecA830. Up-regulation of the heat shock response might have been induced during the initial phase of depletion. Only two genes encoding chaperone-like proteins were negatively affected by SecA830-depletion. The first one was *yabH*, encoding the DnaJ-like protein DjlA. DjlA is anchored in the membrane with a single N-terminal domain preceded by a cytoplasmic segment

containing the DnaJ-like domain (Clarke *et al.*, 1996). The function of the protein remains elusive, but it is thought to interact with membrane sensor kinases probably in conjunction with DnaK (Clarke *et al.*, 1997).

The second negatively affected gene was *htrA*. This gene encodes the periplasmic protein DegP which was present in a reduced amount in SecA-depleted cells. Depending on the temperature DegP can act either as a chaperone or as a protease (for review see Clausen *et al.*, 2002). In contrast to most other heat shock proteins, synthesis of DegP is regulated by σ^E and not σ^{32}. Probably, when fewer proteins were exported into the periplasm in a SecA deficient cell, DegP was less needed and thus expression was down-regulated. Such a negative feedback control is performed by the RseA/B proteins that monitor the level of OMPs in the outer membrane and regulate σ^E activity (de las Peñas *et al.*, 1997).

Taken together, the results of the DNA array analysis presented here constitute a first attempt to track down possible unknown interaction partners of Ffh, FtsY and SecA or to reveal possible regulation networks. The experiments can only mirror the transcription status at a certain time point during growth under depletion conditions. Nevertheless, some interesting connections between the known functions of Ffh, FtsY and SecA and other processes or protein components were found that are worth further investigations.

5.4 Future aims

It was shown here that site-specific proteolysis by TEV protease could be successfully used to inactivate the essential protein SecA *in vivo*. Via biotinylation assays an involvement of SecA in the insertion of two inner membrane proteins could be demonstrated. Therefore it would be interesting to expand this experiment by testing the SecA-dependency of membrane assembly of more structurally different inner membrane proteins. For instance, the PSBT domain could be inserted into the much shorter third or fourth periplasmic loop of MalF or into the periplasmic domains of TolQ, a membrane protein containing 3 transmembrane domains that was found previously to insert into the membrane independently of SecA (Lewin and Webster, 1996). In addition, other secreted proteins like outer membrane proteins or periplasmic substrate binding proteins could be tested as controls in both SecA-depletion and Ffh- or FtsY-depletion assays.

Additional DNA arrays should be carried out, especially to verify the results of the SecA-depletion array analysis (as only 2 replicates were used here). It would be interesting to investigate gene expression profiles of cells only partially depleted of one of the targeting components and compare these to the profiles obtained here after extensive depletion.

The results of the DNA array experiments could be the starting-point to further investigate (a) the role of cellular chaperones in membrane targeting of inner membrane proteins, (b) a putative role of Ffh in flagellar assembly, (c) the role of PspA and/or MsyB in protein translocation, (d) the completely unknown function of PspD and PspE.

In our laboratory a bacterial two-hybrid system has already been successfully used to study protein-protein interactions. An interesting project would be to use this method to find possible new interaction partners of SecA, Ffh and FtsY.

Another project could be to fuse active proteins to the ribosome, either temporarily via the short trigger factor fragment or permanently to the ribosomal proteins near the tunnel exit. For example, by locating SecA in close proximity to the nascent preprotein the question could be clarified if this complex is able to target certain proteins to the inner membrane without the help of additional targeting factors. By permanently fusing TEV-protease to a position near the ribosomal tunnel exit, the cleavage efficiency of nascent-target proteins might be increased.

6 References

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Appendix

A. Characterisation of the *malK* promoter

A.a. Plasmid and strain construction

In order to inactivate SecA-PCS via TEV-protease, the protease had to be regulated differently from the SecA-constructs. One candidate for the regulation of TEV-protease expression was P_{malK}. This divergent promoter controls the expression of the operons *malK-lamB-malT* and *malEFG* of the maltose system. The *malK* gene encodes the ATP-binding component of the maltose ABC transporter.

Plasmid pMIC16 carries the reporter gene *phoA* behind P_{malK} as well as the *lacZ* and *bla* genes (Braun, 1996). Via restriction sites *Stu I* and *Xho I* the spare gene *lacZ* was cut out and the remaining vector was religated (Fig. A.1). It turned out that *phoA* was not in the correct reading frame of the P_{malK} promoter. Therefore, a PCR was performed with primers fixexpressleft and fixexpressright. Primer fixexpressright contained a *Nco I* site that included a start codon and a repositioned *BamH I* site. By inserting the PCR product into *BamH I* and *Pst I* sites the *BamH I* restriction site was moved in a position that shifted the *phoA*-orf into the correct reading frame. The resulting plasmid was renamed pTH2 and inserted into strain TH2 by TSS-transformation.

Strain TH2 is a derivative of strain DHB3 (Δ*phoA*, Δ*malF* and *malI*::Tn*10*) and was constructed via P1 transduction to introduce a chloramphenicol resistance gene together with a *treR*::Tn*10* fragment. This strain provided the background for P_{malK} Regulation (Fig. A.1). In the absence of *malF,* maltose is not actively transported into the cell but can enter the cell via diffusion through TreB-channels. In this way the amount of inducer can be regulated. To increase the number of channels the repressor TreR, that negatively regulates the expression of *treB* was inactivated by introducing the *treR*::Tn*10* fragment. In addition, also the repressor of the *malXY* operon *malI* was inactivated in this strain. As *malY* is a repressor of the general *mal*-system activator MalT, inactivation of *malI* reduces the amount of MalT. In this manner P_{malK} was repressed by a factor of 10 to increase the sensitivity of induction.

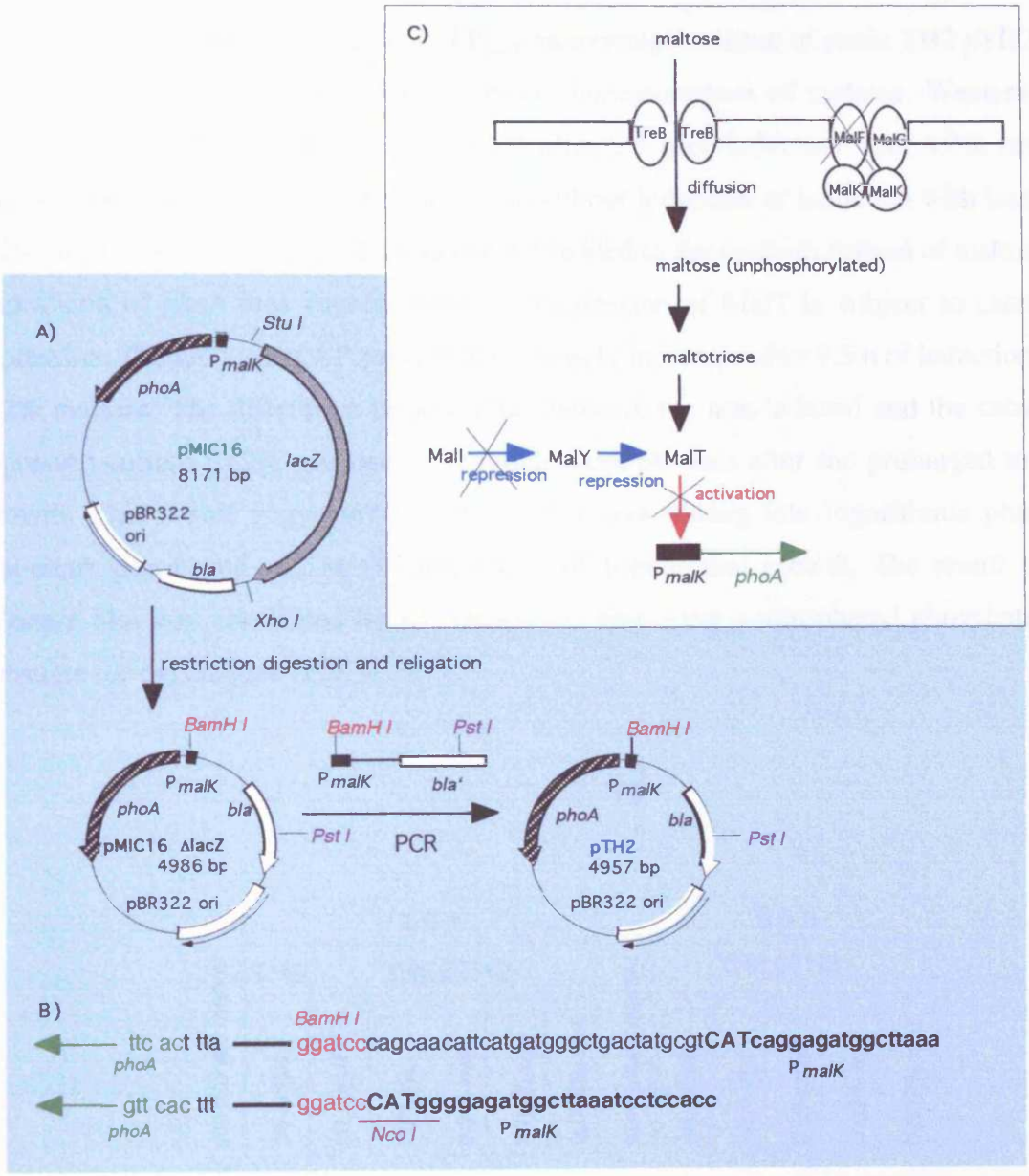

Fig. A.1 A) Construction of pTH2 and shifting of the *phoA* orf into the correct reading frame via PCR. B) Detail of the start and downstream region of P_{malK} before and after the shift into the correct reading frame. C) Induction system of P_{malK} in strain TH2.

A.b. Gene expression and AP- activity tests

To test the expression of *phoA* behind P_{malK} an overnight culture of strain TH2 pTH2 was diluted in LB medium containing different concentrations of maltose. Western blot analysis with α-AP antibody showed that after 2.5 h of induction with 0.2% maltose slightly more protein was synthesised than without induction or induction with less than 0.2% maltose (Fig. A.2). If 0.2% glucose was added to the medium instead of maltose the expression of *phoA* was repressed as the expression of MalT is subject to catabolite repression. The amount of AP-protein was strongly increased after 9.5 h of induction with 0.2% maltose. The difference between the induced, the non-induced and the catabolite repressed culture (0.2% glucose) was much more obvious after the prolonged time of growth. This result suggested that P_{malK} is active during late logarithmic phase or stationary phase and not at the beginning of logarithmic growth. The result of the Western blot was confirmed by an AP-activity test using p-nitrophenyl phosphate as a substrate for the enzyme (Fig. A.3).

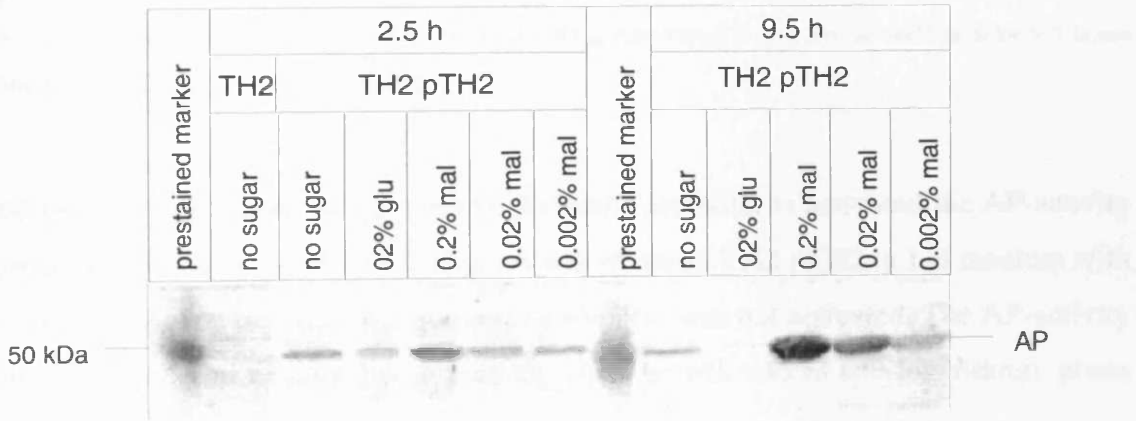

Fig. A.2 Westernblot analysis with α-AP antibody from a 10% SDS-PAGE. Overnight cultures of strains TH2 and TH2 pTH2 were diluted 1/20 in LB medium containing 30 µg/ml chloramphenicol and 100 µg/ml ampicillin. Different concentrations of maltose were added to induce expression of *phoA*. Glucose was added to repress *phoA* expression. Samples for SDS-PAGE were taken 2.5 hours after dilution at an OD_{578} between 0.5 and 0.6, and 9.5 hours after dilution at an OD_{578} between 2.7 and 2.9.

added to repress *phoA* expression. Samples for SDS-PAGE were taken 2.5 hours after dilution at an OD_{578} between 0.5 and 0.6, and 9.5 hours after dilution at an OD_{578} between 2.7 and 2.9.

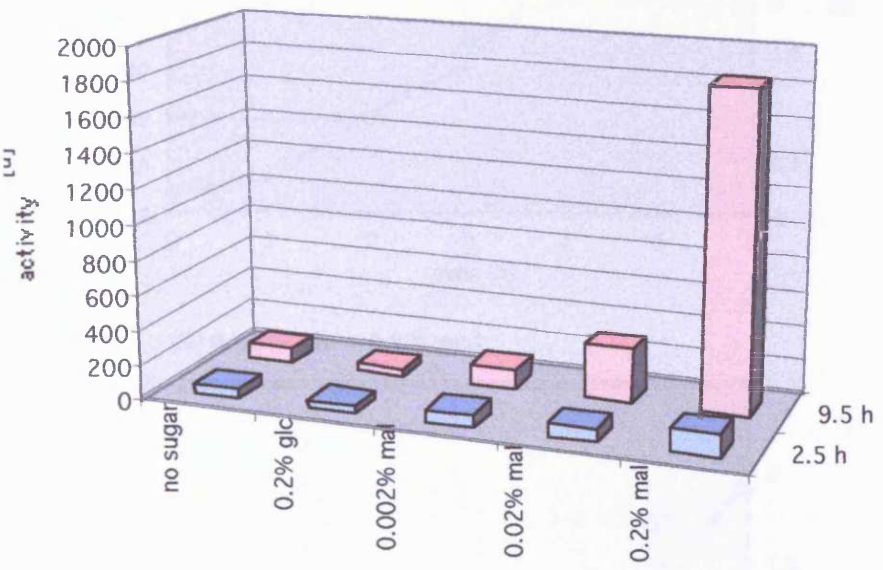

Fig. A.3 AP-activity in strain TH2 pTH2. An overnight culture was diluted 1/20 in LB medium containing 100 µg/ml ampicillin and different concentrations of maltose or 0.2% glucose respectively. Samples for the activity test were taken 2.5 hours after dilution at an OD_{578} between 0.5 and 0.6, as well as after 9.5 hours after dilution at an OD_{578} between 2.7 and 2.9.

To find out at which phase during growth the *malK* promoter is activated the AP-activity was measured in hourly intervalls during growth of strain TH2 pTH2 in LB medium with 0.2% maltose. During the first two hours the promoter was not activated. The AP-activity then increased but had not reached a plateau when growth was in late logarithmic phase or early statinory phase (Fig. A.4).

When 0.05% glucose was added to the medium in addition to 0.2% maltose the increase of the AP-activity was delayed. This delay was caused by catabolite repression of the maltose-system. When all glucose was metabolised the AP-activity increased more steeply than in the first experiment where no glucose was added. When a higher concentration of glucose (0.2%) was added in addition to 0.2% maltose, the catabolite

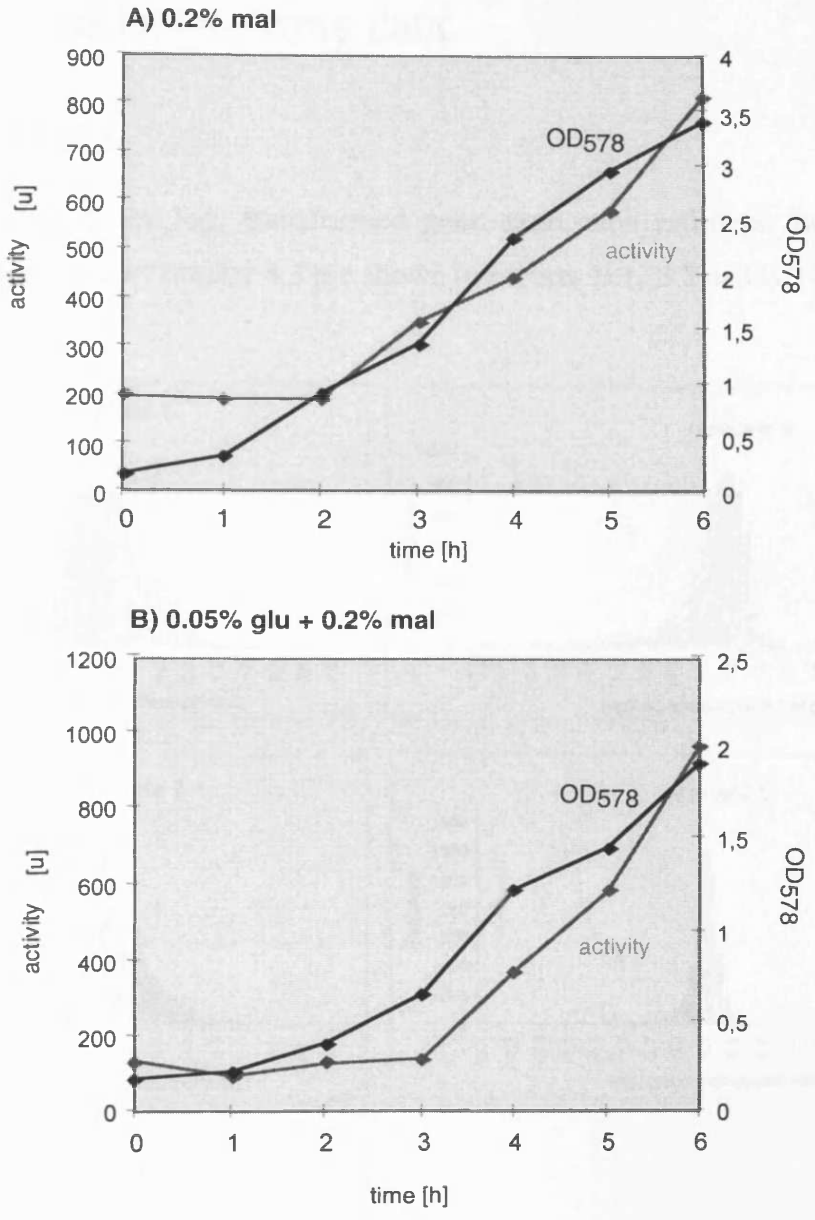

Fig. A.4 AP-activity in strain TH2 pTH2 and OD_{578}. An overnight culture of strain TH2 pTH2 was diluted 1/20 in LB 100 µg/ml ampicillin and A) 0.2% maltose or B) 0.05% glucose and 0.2% maltose.

Due to the late activation of P_{malK}, this promoter was unsuitable for the specific induction of TEV-protease in a SecA-inactivation system. Nevertheless, late induction of gene expression might be very useful in other experimental systems. For example, P_{malK}, might provide an interesting solution to reduce the culture volume to gain an appropriate amount of protein for purification.

B. Additional DNA-Array data

B.a. Histograms

The distributions of the $\log_2$ transformed gene expression ratios of the DNA-array experiments described in chapter 4.3 are shown in Figures B.1, B.2 and B.3 below.

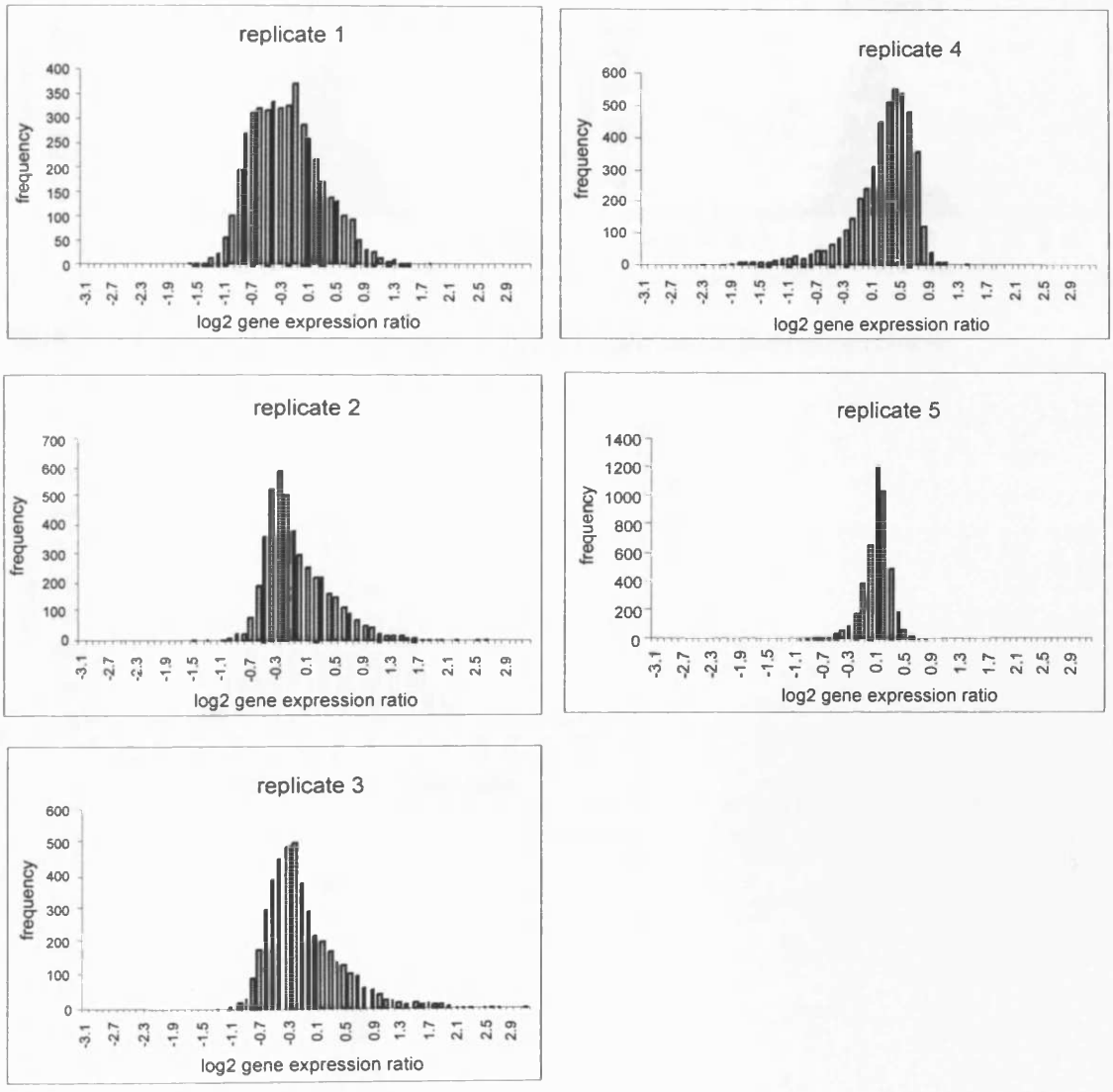

Fig. B.1 Histograms of the five replicates of the Ffh-depletion DNA-array experiments.

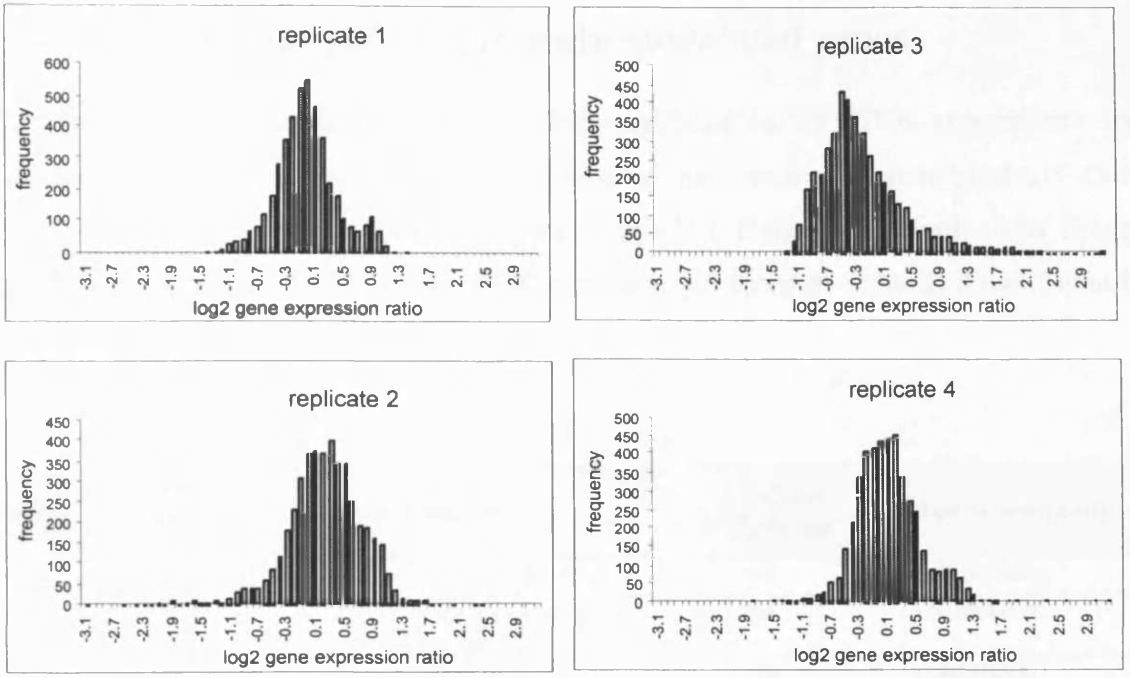

Fig. B.2 Histograms of the four replicates of the FtsY-depletion DNA-array experiments.

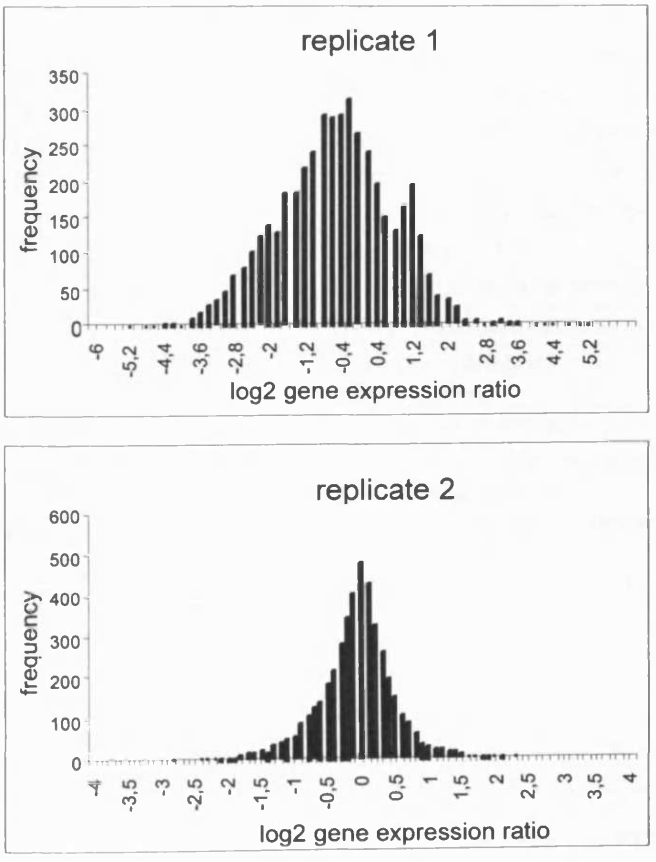

Fig. B.3 Histograms of the two replicates of the SecA830-depletion DNA-array experiments.

B.b. Classification of significantly modulated genes

Genes that were significantly up- or down-regulated in the DNA-experiments were divided into groups according to the function of their respective gene products. Genes affected by the depletion of Ffh are listed in Table B.1. Genes with significantly changed gene expression after FtsY- or SecA830-depletion are listed in Table B.2 and Table B.3 respectively.

Gene	Gene location (direction)	Gene product	Protein location	Functional group
ftsN (e)	88.81 (-)	cell division protein FtsN	IM	cell division
tsr	98.92 (+)	CheD, methylaccepting chemotaxis protein I	IM	chemotaxis
cspA	80.14 (+)	cold shock protein CspA	CP	cold shock
arsB	78.60 (+)	arsenical pump membrane protein	IM	export
fliA	43.09 (-)	RNA polymerase sigma factor for flagellar operon	CP	flagellar assembly
fliC	43.11 (-)	flagellin	flagellar	flagellar assembly
flgB	24.36 (+)	flagellar basal-body rod protein	flagellar, PP	flagellar assembly
flgE	24.40 (+)	flagellar hook protein	flagellar	flagellar assembly
flgF	24.42 (+)	flagellar basal-body rod protein	flagellar, PP	flagellar assembly
flgG	24.44 (+)	flagellar basal-body rod protein	flagellar, PP	flagellar assembly
clpB	58.84 (-)	ClpB, heat shock protein F84.1	CP	heat shock
dnaK	0.26 (+)	DnaK, chaperone	CP	heat shock
hslS	83.29 (-)	16 kDa heat shock protein B	CP	heat shock
hslT	83.30 (-)	16 kDa heat shock protein A	CP	heat shock
glpD	76.73 (+)	glycerol-3-phosphat dehydrogenase	CP	metabolism
araA	1.44 (-)	L-arabinose isomerase	CP	metabolism
eno	62.61 (-)	enolase, glycolysis, also involved in RNA degradation	CP	metabolism
fba	66.14 (-)	fructose bisphosphate aldolase class II, glycolysis	CP	metabolism
narG	27.57 (+)	nitrate reductase alpha chain	IM associated	metabolism
pgK	66.16 (-)	phosphoglycerate kinase, glycolysis	CP	metabolism
tktA	66.34 (-)	transketolase1	CP	metabolism
lysU	93.78 (-)	lysyl-tRNA-synthatase, heat inducible	CP	translation
rplJ	90.05 (+)	50S ribosomal protein L10, translational repressor of rplJL-rpoBC operon	CP	translation
rplL	90.06 (+)	50S ribosomal protein L7/ L12	CP	translation

rplY	49.16 (+)	50S ribosomal protein L25	CP	translation
tufB	89.96 (+)	elongation factor Tu	CP and IM associated	translation
ompA	21.95 (-)	outer membrane protein A, porin	OM	transport
yhcN	72.93 (+)	hypothetical 11.2 kDa protein in argR-cafA intergenic region	unknown	unknown
ynaJ	30.07 (+)	hypothetical 9.3 kDa protein in tpx-fnr intergenic region	potential IM	unknown

Tab. B.1 Classification of genes affected by Ffh-depletion. Positions for gene location on the *E. coli* K-12 linkage map in minutes were taken from http://genolist.pasteur.fr/colibri/index.html. Informations about gene products and functions were obtained from http://www.genome.ad.jp/dbget-bin7get_htext?E.coli.kegg+-f+T+W+B and http://genproduct.mbl.edu. Up-regulated genes are drawn in red, down-regulated genes in blue. Abbreviations: CP = cytoplasm, IM = inner membrane, PP = periplasm, OM = outermembrane, (e) = essential gene.

Gene	Gene location (direction)	Gene product	Protein location	Functional group
hflB (e)	71.62 (-)	FtsH, ATP-dependent zinc metallopeptidase, involved in the degradation of sigma-32, cell growth, septum formation, phage lambda development , acting on SecY	IM	cell division
cspE	14.15 (+)	cold shock-like protein	CP	cold shock
clpB	58.84 (-)	ClpB, heat shock protein F84.1	CP	heat shock
dnaK	0.26 (+)	DnaK, chaperone	CP	heat shock
hslS	83.29 (-)	16 kDa heat shock protein B	CP	heat shock
hslT	83.30 (-)	16 kDa heat shock protein A	CP	heat shock
hslV	88.79 (-)	HslV, protease subunit	CP	heat shock
htpG	10.66 (+)	HtpG, chaperone	CP	heat shock
htpX	41.16 (-)	probable protease HtpX	IM	heat shock
mopA (e)	94.17 (+)	GroEL, 60 kDa chaperonin	CP	heat shock
mopB (e)	94.16 (+)	GroES, 10 kDa chaperonin	CP	heat shock
cynT	7.72 (+)	carbonic anhydrase	CP	metabolism
glpD	76.73 (+)	glycerol-3-phosphat dehydrogenase	CP	metabolism
glpK	88.66 (-)	glycerol kinase	CP	metabolism
gltA	16.22 (-)	citrate synthase	CP	metabolism
nanA	72.65 (-)	N-acetylneuraminate-lyase	CP	metabolism
nanK	72.58 (-)	glucokinase, sialic acid utilisation, amino sugar conversion	CP	metabolism
narY	33.09 (-)	nitrate reductase, cryptic NRII beta subunit	IM	metabolism

rpiB	92.92 (+)	ribose-5-phospate isomerase B	CP	metabolism
sdhB	16.32 (+)	succinate dehydrogenase iron-sulphur protein	CP	metabolism
sucD	16.46 (+)	succinyl-CoA syntheatse alpha-chain	CP	metabolism
tnaA	83.77 (+)	tryptophanase	CP	metabolism
tnaL	83.77 (+)	tryptophanase leader peptide	CP	metabolism
yjhC	97.06 (+)	hyp. 41.9 kDa protein in leuX-fecE intergenic region, KpLE2 phage like element, putative NAD(P) binding dehydrogenase	CP	phage related functions
lysU	93.78 (-)	lysyl-tRNA-synthetase, heat inducible	CP	translation
rplI	95.35 (+)	50S ribosomal protein L9	CP	translation
rplJ	90.05 (+)	50S ribosomal protein L10	CP	translation
rplL	90.06 (+)	50S ribosomal protein L7/L12	CP	translation
gatA	46.83 (-)	galactitol-specific enzyme IIA of PTS system	CP	transport
nanT	72.61 (-)	putative sialic acid transporter, MFS family, amino sugar conversion	IM	transport
nikD	77.92 (+)	ATP-binding component of ABC transporter for nickel	CP	transport
ompX	18.32 (+)	outer membrane protein, porin	OM	transport
yjhB	97.03 (+)	hypothetical 46.6 kda protein in leuX-fecE intergenic region, hypothetical metabolite transport protein, belongs to the MFS transport family, strong homology to nanT, KpLE2 phage like element	potential IM	transport
trmD	59.12 (-)	tRNA (guanine-7)-methyltransferase	CP	tRNA-modification
yacG	2.41 (-)	hypothetical 5.8 kDa protein in mutT-guaC intergenic region	unknown	unknown
yaiW	8.56 (+)	hypothetical 40.4 kDa protein in sbmA-ddlA intergenic region	unknown	unknown
yhcH	72.57 (-)	hypothetical 17 kDa protein in gltF-nanT intergenic region	unknown	unknown
yjhA	97.78 (-)	conserved hypothetical protein with outer membrane domain	unknown	unknown

Tab. B.2 Classification of genes affected by FtsY-depletion. Positions for gene location on the *E. coli* K-12 linkage map in minutes were taken from http://genolist.pasteur.fr/colibri/index.html. Informations about gene products and functions were obtained from http://www.genome.ad.jp/dbget-bin7get_htext?E.coli.kegg+-f+T+W+B and http://genproduct.mbl.edu. Up-regulated genes are drawn in red, down-regulated genes in blue. Abbreviations: CP = cytoplasm, IM = inner membrane, PP = periplasm, OM = outermembrane, (e) = essential gene.

Gene	Gene location (direction)	Gene product	Protein location	Functional group
rspA	35.61 (-)	starvation sensing protein, repressor of sigma S	CP?	cell processes
ygfL	65.04 (+)	Ssna protein, involved in decline of cell viability	?	cell processes
mrdB	14.32 (-9	rod-shape determining protein	IM	cell shape
folK	3.39 (-)	2-amino-4-hydroxy-6-hydroxy methyldihydropteridine pyrophosphokinase	CP	cofactor biosynthesis
nadC	2.54 (-)	nicotinate-nucleotide pyrophosphorylase, NAD biosynthesis	CP	cofactor biosynthesis
ssuD	21.43 (-)	alkanesulfonate monooxygenase	CP?	cofactor biosynthesis
deaD	71.21 (-9	ATP-dependent RNA helicase, cold-shock DEAD-box protein A	CP	cold shock, cell division
hofD	74.65 (+)	general secretion pathway, methyl transferase	IM	export
hofG	74.55 (+)	putative general secretion pathway prot.across OM	?	export
mdfA	19.03 (+)	multidrug translocase, cm resistance	IM	export
msyB	23.99 (-)	acidic protein MsyB, could participate in protein export	unknown	export?
yhiV		hyp. 111.5 kD prot. in hdeD-gadA interg. reg., potential drug efflux pump	potential IM	export
htrA (e)	3.90 (+)	DegP, protease/chaperone	PP	heat shock
aceA	90.85 (+)	isocitrate lyase	CP	metabolism
adhE	27.91 (-)	aldehyde-alcohol dehydrogenase	CP	metabolism
adi	93.46 (-)	biodegradative arginine decarboxylase	CP	metabolism
agaS	70.69 (+)	putative tagatose-6-phosphate ketose	CP?	metabolism
cysQ	95.58 (+)	CysQ protein	IM associated	metabolism
ebgA	69.41 (+)	evolved beta-galactosidase subunit	CP?	metabolism
fucI	63.24 (+)	L-fucose isomerase	CP	metabolism
fucK	63.28 (+)	L-fuculose kinase	CP?	metabolism
glgS	68.76 (-)	glycogen synthesis protein, growth-phase regulated, rpoS dependent	CP	metabolism
gltD	72.36 (+)	glutamate synthase, small subunit	CP	metabolism
hycG	61.25 (-)	hydrogenase 3 subunit	IM	metabolism
maeB	55.49 (-)	putative NADP+ linked malic enzyme	CP?	metabolism
mtlD	81.31 (+)	mannitol-1-phosphate dehydrogenase	CP?	metabolism
nrfG	92.50 (+)	formate dependent nitrite reductase subunit	CP?	metabolism

pepB	57.19 (-)	peptidase B	CP	metabolism
pepE	91.12 (-)	peptidase E	CP	metabolism
serB	99.64 (+)	phosphoserine phosphatase	CP	metabolism
uxaC	69.86 (-)	uronate isomerase	CP?	metabolism
uxuB	98.09 (+)	D-mannonate oxidoreductase	CP?	metabolism
yafH	5.19 (-)	putative acyl-CoA dehydrogenase	?	metabolism
yfaU	50.79 (-)	putative aldolase	CP?	metabolism
yfhT	57.47 (-)	hca operon transcriptional activator	CP	metabolism
yhaE	70.48 (-)	tatronate semialdehyde reductase	CP?	metabolism
yhaF	70.50 (-)	alpha-dehydro-beta-deoxy D-glucarate aldolase	CP?	metabolism
yheB	74.69 (-)	probable bifunctional chitinase/lysozyme	PP	metabolism
yhfW	75.58 (-)	putative phosphoentomutase	CP?	metabolism
yidU	83.41 (-)	2-oxo-3-deoxygalactonate 6-phosphate aldolase	CP?	metabolism
bisZ	42.09 (-)	trimethylamine-N-oxide reductase 2	CP	metabolism/ electron acceptor
dmsA	20.27 (+)	anaerobic dimethyl sulfoxide reductase chain A	IM associated	metabolism/ electron acceptor
frdB	94.36 (-)	fumarate reductase iron-sulphur protein	CP?	metabolism/ electron acceptor
hybO	67.75 (-)	hydrogenase 2, small subunit	IM	metabolism/ electron donor
amiA	54.97 (+)	probable N-acetylmuramoyl L-alanine amidase	?	metabolism/ cell envelope
nlpD	61.77 (-)	lipoprotein, may be involved in stationary phase survival, may function in cell wall formation	attached to IM	metabolism/ cell envelope
rfc	45.35 (-)	O-antigen polymerase	IM	metabolism/ cell envelope
wzb	45.99 (-)	involved in lipopolysaccharide biosynthesis	?	metabolism/ cell envelope
wzzE	85.50 (+)	lipopolysaccharide biosynthesis protein	potential IM	metabolism/ cell envelope
ybeG	14.14 (+)	CrcA, palmitoyl transferase for lipid A	OM	metabolism/ cell envelope
ybhC	17.36 (-)	putative pectinesterase, putative lipoprotein	OM attached by lipid anchor	metabolism/ cell envelope
ascG	61.14 (-)	cryptic asc operon repressor, PTS system	CP	metabolism/ regulation
aqpZ	19.71 (-)	aquaporin Z	IM	osmoregulation
mdoH	23.93 (+)	periplasmic glucans biosynthesis protein	IM	osmoregulation
intE	25.84 (-)	prophage lambda integrase	CP	phage related functions
intR	30.39 (-)	putative lambdoid prophage Rac integrase	CP	phage related function
nohB	12.50 (+)	prophage QSR' DNA packaging protein NU1 homolog	CP	phage related function

pspA	29.45 (+)	phage shock protein A, negative regulator, unknoen function in the maintenance of pmf	IM-associated	phage shock
pspB	29.46 (+)	phage shock protein, transcription regulator	IM	phage shock
pspC	29.47 (+)	phage shock protein C, transcription regulator with pspB	IM	phage shock
pspD	29.48 (+)	phage shock protein D	CP	phage shock
pspE	29.48 (+)	phage shock protein E	CP	phage shock
recG	82.40 (+)	ATP-dependent DNA helicase	CP?	recombination
recQ	86.30 (+)	ATP-dependent helicase	CP	recombination
yigN	86.54 (+)	hyp. 54.7 kDa prot. in UDP-ubiE intergenic reg.,involved in DNA recombination	CP?	recombination
chaC	27.41 (+)	cation transport transcription regulator	CP	regulation
ycgE	26.14 (-)	hyp. transcriptional regulator in icdc-minE interg. reg.	CP?	regulation
ydjF	39.92 (-)	putative DEOR-type transcriptional regulator	CP?	regulation
yfaX	50.86 (-)	putative transcriptional regulator	CP?	regulation
ygbI	61.62 (-)	hyp. transcriptional regulator in mutS-rpoS intergenic region	CP?	regulation
dnaB	91.87 (+)	replicative DNA helicase	CP	replication
dnaE	4.42 (+)	DNA polymerase III alpha subunit	CP	replication
holD	99.27 (+)	DNA-polymerase, psi-subunit	CP	replication
hrpB	3.49 (+)	ATP-dependent helicase	CP	replication
pcnB	3.40 (-)	poly(A) polymerase	CP	replication
hydG	90.55 (+)	transcription regulator ZraR, activated by ZraS	CP	signal transduction
hydH	90.52 (+)	sensor protein ZraS	IM	signal transduction
kdpD	15.54 (-)	sensor protein, phosphorylates KdpE	IM	signal transduction
qseC	68.30 (+)	probable sensor protein, activates flagellar regulon	potential IM	signal transduction
rstB	36.23 (+)	sensor histidine kinase	IM	signal transduction
yfhK	57.93	probable sensor protein YfhK, may activate YfhA	potential IM	signal transduction
cysS	11.94 (+)	cysteinyl-tRNA synthetase	CP	translation
glyS	80.19 (-)	glycyl-tRNA synthetase beta chain	CP	translation
infC (e)	38.76 (-)	translation initiation factor IF-3	CP	translation
pheS	38.71 8-)	phenylalanyl-tRNA synthetase alpha chain	CP	translation
priB	95.34 (+)	primosomal replication protein	CP	translation
rimL	32.27 (+)	ribosomal-protein-serine acetyltransferase	CP	translation
rplI	95.35 (+)	50S ribosomal protein L9, binds to 23S RNA	CP	translation

rplP	74.29 (-)	50S ribosomal subunit protein L16	PC	translation
rpsQ	74.28 (-)	30S ribosomal prot. S17	CP	translation
rpsR	95.35 (+)	30S ribosomal prot. S18	CP	translation
aroP	2.59 (-)	aromatic amino acid transport protein, TyrR regulon	IM	transport
dppB	79.81 (-)	dipeptide transport system permease protein, ABC transport	IM	transport
dsdX	53.37 (+)	probable permease for an unknown substrate	potential IM	transport
fecE	97.18 8-)	iron(III) dicitrate transport ATP - binding protein	IM associated	transport
feoB	76.26 (+)	ferrous iron uptake	IM	transport
fhuA	3.61 (+)	receptor for ferrichrome	OM	transport
fhuC	3.66 (+)	ferrichrome-iron transport system ATP-binding protein	IM associated	transport
fhuE	24.97 (-)	receptor for ferric-rhodotorulic acid	OM	transport
fruA	48.67 (-)	PTS system, fructose specific IIBC component	IM	transport
gltS	82.45 (-)	glutamate permease	IM	transport
gntP	98.02 (-)	high-affinity gluconate transporter	IM	transport
gntT	76.40 (+)	high-affinity gluconate transporter	IM	transport
gntU_2	77.03 (-)	low-affinity gluconate transporter	IM	transport
livG	77.41 (-)	leucine/isoleucine/valine transport system ATP-binding protein	PP	transport
livH	77.45 (-)	high affinity branched chain amino acid transport protein (ABC)	IM	transport
manX	40.96 (+)	PTS system, mannose specific IIAB component	CP	transport
mtr	71.18 (-)	tryptophane specific transport protein	IM	transport
phnK	93.02 (-)	putative phosphanate transport protein (ABC)	CP	transport
sdaC	63.08 (+)	serine transporter	potential IM	transport
sgcC	97.57 (-)	putative phosphotransferase enzyme IIC	IM	transport
tdcC	70.30 (-)	threonine transporter, symport system	potential IM	transport
treB	96.19 (-)	PTS system, trehalose specific IIBC component	IM	transport
ybdA	13.40 (+)	putative transport protein (POT family)	IM	transport
yeiJ	48.52 (-)	43.4 kDa prot. in nfo-fruA interg. reg., hyp. transport protein	potential IM	transport
ygcS	62.39 (-)	putative metabolite transport protein (MFS family), porter	potential IM	transport
ygdQ	63.99 (+)	putative transport prot.	potential IM	transport
ygiS	68.20 (-)	oligopeptide transport substrate-binding protein	potential PP	transport
yhdW	73.65 (+)	putative polar amino acid	potential	transport

		transport system substrate-binding	PP	
yhdX	73.67 (+)	hyp. amino acid ABC transporter permease	potential IM	transport
yhdY	73.70 (+)	putative polar amino acid transport system permease protein, ABC	IM	transport
yiaO	80.70 (+)	putative periplasmic component of transport system	PP	transport
yjcX	92.88 (-)	allose binding protein (ABC)	PP	transport
yjdE	93.41 (-)	hyp. transport protein	potential IM	transport
yjdL	93.82 (-)	putative di-/tripeptide transport protein, proton-dependent (POT family)	IM	transport
mppA	29.99 (+)	periplasmic murein peptide-binding protein	PP	transport /cell wall
ydcR	32.51 (+)	putative transcription regulator, also putative ATP-binding component of ABC transporter	IM-Associated?	transport/ regulation
b0299	extrachr.	transposase insF for IS3	CP?	transposon related function
tra8_1	extrachr.	transposase for IS30	CP?	transposon related function
gutQ	60.96 (+)	putative ATP binding protein	unknown	unknown
hdeB	78.75 (-)	10 kDa protein, rpoS-dependent	PP	unknown
mbhA	5.39 (+)	unknown	unknown	unknown
yaaF	0.59 (+)	hyp. 32.6 kDa prot. in lytB-dapB intergenic reg.	unknown	unknown
yacH	2.79 (-)	putative membrane protein	IM	unknown
yadC	3.23 (-)	hyp. fimbrial-like prot. in panB-htrE interg. reg.	unknown	unknown
yaiA	8.76 (+)	hyp. 7.3 kDa prot. in aroL-aroM intergenic region	unknown	unknown
yaiX	8.19 (-)	unknown, interrupted by IS2A	unknown	unknown
ybeK	14.72 (-)	hyp. 33.8 kD prot. in leuS-gltL interg. region	unknown	unknown
ybhE	17.20 (+)	putative isomerase	unknown	unknown
ybjE	19.68 (-)	hyp. 34.4 kDa prot. in poxB-aqpZ interg. reg.	potential IM	unknown
yccS	22.01 (-)	hyp. 82 kDa prot. in sulA-helD interg. reg.	potential IM	unknown
ycdJ	23.07 (-)	putative hydrolase	unknown	unknown
ydeH	34.94 (-)	hyp. 33.9 kDa prot. in marB-dcp intergenic region	unknown	unknown
yfbS	51.90 (-)	hyp. 65.9 kD protein in lrhA-ackA interg. reg.	potential IM	unknown
ygcP	62.34 (+)	hyp. 20.8 kD prot. in cysJ-eno inter. reg.	unknown	unknown
yhdH	73.31 (+)	hyp. 34.7 kD prot. in mreB-accB interg.reg.	unknown	unknown
yhgE	76.06 (-)	hyp. 64.6 kD prot. in mrcA-pckA interg. reg.	potential IM	unknown
yhhS	77.78 (-)	hyp. 43.8 kD protein in ftsY-nikA	potential IM	unknown

		interg. reg.		
yhjC	79.11 (+)	hyp. transcriptional regulator in treF-kdgK interg. reg.	unknown	unknown
yiaV	80.85 (-)	hyp. 41.8 kD protein in avtA-selB interg. Reg.	trans-membrane	unknown
yicC	82.22 (+)	33.2 kDa prot.in dinD-rph intergenic reg.	unknown	unknown
yicE	82.48 (+)	hyp. 48.9 kDa prot. in gltS-selC intergenic reg.	potential IM	unknown
yidJ	83.09 (-)	putative sulfatase	unknown	unknown
yieO	84.86 (-)	hyp. 51.5 kD prot. in rbsR-rrsC interg. reg.	IM	unknown
yijC	89.64 (+)	hyp. 26.6 kDa prot. in udhA-trmA intergenic reg., belongs to tetR/acrR transcription regulators	CP	unknown
yijP	89.37 (-)	hyp. 30.5 kDa prot. in dnaT-bglJ interg. reg.	potential IM	unknown
yjhA	97.78 (-)	hyp. 28.3 kD prot. in fecI-fimB interg. reg.	unknown	unknown
yqgA	66.99 (+)	hyp. 24.6 kD prot. in speC-glcB interg. Reg.	potential IM	unknown
yqjG	70.03 (+)	hyp. 37.4 kDa prot. in exuR-tdcC interg. reg.	unknown	unknown
yraJ	70.84 (+)	hyp. outer membrane usher protein in agaI-mtr intergenic region	OM	unknown
yabH	1.24 (+)	DjlA, proposed to dock and interact with a variety of membrane proteins, DnaJ-like co-chaperone of DnaK	IM	unknown/ chaperone

Tab. B.3 Classification of genes affected by SecA830-depletion. Positions for gene location on the *E. coli* K-12 linkage map (in minutes) were taken from http://genolist.pasteur.fr/colibri/index.html. Informations about gene products and functions were obtained from http://www.genome.ad.jp/dbget-bin7get_htext?E.coli.kegg+-f+T+W+B and http://genproduct.mbl.edu. Up-regulated genes are drawn in red, down-regulated genes in blue. Abbreviations: CP = cytoplasm, IM = inner membrane, PP = periplasm, OM = outermembrane, (e) = essential gene.

C. Abbreviations

aa	amino acid
ADP	adenosine diphosphate
Amp	ampere / ampicillin
AP	alkaline phosphatase
APS	ammoniumperoxydisulfide
ara	arabinose
aTc	anhydrotetracycline
ATP	adenosine triphosphate
att	attachment
bp	basepair
BSA	bovine serum albumin
CIAP	calf intestine alkaline phosphatase
CP	cytoplasm
Cm	chloramphenicol
Da	Dalton
dH_2O	distilled water
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetatic acid
ER	endoplasmatic reticulum
GTP	guanine triphosphate
glu	glucose
h	hour
hyp.	Hypothetical
IM	inner membrane
interg.	intergenic
IPTG	Isopropyl b-D thiogalactoside
k	kilo
Kan	kanamycin
kb	kilobasepair
LB	Luria Bertani media
LPS	lipopolysaccharide

M	molar
m	milli
mal	maltose
NBT	4-nitroblue-tetrazoliumchloride
nm	nanometre
nt	nucleotide
OD	optical density
OM	outer membrane
ori	origin of replication
P_i	organic phosphate
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PCS	protease cleavage site
PMF	proton motive force
pNPP	p-nitrophenyl phosphate
PP	periplasm
prot.	protein
R	resistance
reg.	region
RNA	Ribonucleic acid
rpm	rounds per minute
RT	room temperature
SD	Shine-Dalgarno
SDS	sodiumdodecylsulfate
Spec	spectinomycin
SRP	signal recognition factor
Tc	tetracycline
TEMED	N,N,N',N'-tetramethylendiamin
TEV	Tabacco etch virus
tet	tetracycline
TF	trigger factor
TRIS	tris hydroxymethylaminmethan
ts	temperature sensitive
u	unit
UV	ultra violet
V	volt
v/v	volume per volume

wt	wild-type
w/v	weight per volume
X	any amino acid
X-GLUC	5-bromo-4-chloro-3-indolyl-β-D-glucoronide
XP	5-bromo-4-chloro-3-indolylphosphat