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A C A D E M I C D I S S E R T A T I O N

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CONTENTS

ABBREVIATIONS

aa	amino acid
AAA^+	ATPase associated proteins with various cellular activities
ADP	adenosine monophosphate
AmyL	α-amylase of *Bacillus licheniformis*
AmyQ	α-amylase of *Bacillus amyloliquefaciens*
AmyS	α-amylase of *Bacillus stearothermophilus*
AP	alkaline phosphatase
ACP	acyl carrier protein
ATP	adenosine triphosphate
BglA	β-glucanase
BlaP	β-lactamase of *B. licheniformis*
CIRCE	controlling inverted repeat of chaperone expression
CS	citrate synthase
CsA	cyclosporin A
Cyp	cyclophilin
C-terminal	carboxyl-terminal
CWBP	cell wall-bound protein
D-ala	D-alanyl ester
3D	three-dimensional
Dcl	D-alanyl carrier protein ligase
Dcp	D-alanyl acyl carrier protein
DT	diphteria toxoid
ER	endoplasmic reticulum
Gro-P	glycerol phosphate
Hsp	heat shock protein
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
LTA	lipoteichoic acid
Mip	macrophage infectivity potentiator
N-terminal	amino-terminal
PA	protective antigen of *B. anthracis*
PBP	penicillin binding protein
Peh	endopolygalacturonase
Pme	pectin methylesterase
PMF	proton motive force
Pnl	pneumolysin
PPIase	peptidyl prolyl *cis/trans* isomerase
RNase T1	ribonuclease T1
S1	pertussis toxin subunit 1
S4	pertussis toxin subunit 4
Sak	staphylokinase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP	signal peptidase
SRP	signal recognition particle
TEM-1	TEM-1 β-lactamase of *E. coli*
TF	trigger factor
TPR	tetratricopeptide repeat
wt	wild type
WTA	wall teichoic acid

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Vitikainen M**., Lappalainen I., Seppala R., Antelmann H., Boer H., Taira S., Savilahti H., Hecker M., Vihinen M., Sarvas M. and Kontinen V.P. (2004). Structure-function analysis of PrsA reveals essential roles for the parvulin-like and flanking N- and C-terminal domains in protein folding and secretion in *Bacillus subtilis*. *J Biol Chem* **279**:19302-19314.

II **Vitikainen M**., Pummi T., Airaksinen U., Wahlström E., Wu H., Sarvas M. and Kontinen V.P. (2001). Quantitation of the capacity of the secretion apparatus and requirement for PrsA in growth and secretion of α-amylase in *Bacillus subtilis*. *J Bacteriol* **183**:1881-1890.

III **Vitikainen M**., Hyyryläinen H., Kivimäki A., Kontinen V.P. and Sarvas M. (2004). Secretion of heterologous proteins in *Bacillus subtilis* can be improved by engineering cell components affecting posttranslocational protein folding and degradation. *Manuscript* submitted.

ABSTRACT

Protein folding is crucial for proteins in order to gain their correct three-dimensional conformation and become functionally active. In most cases this folding process needs the assistance of chaperones and folding catalysts. In this thesis the posttranslocational folding of secretory proteins in *Bacillus subtilis* was studied, namely the folding of exoproteins once they have been translocated across the cytoplasmic membrane into the membrane-cell wall interface and need to fold correctly in this environment. A special focus was on the role of PrsA lipoprotein in this posttranslocational folding process. The aim was to further characterize the PrsA protein with a structure-function analysis and to study its role as a limiting factor in secretion.

PrsA protein was essential for viability of cells; its depletion resulted in abnormal filamentous growth eventually leading to cell lysis. PrsA was an abundant protein on the outer surface of the cell membrane. The number of PrsA molecules per cell was estimated to be about 20 000 molecules, yet only a few hundred molecules per cell were enough to support normal growth.

PrsA exhibited an enzymatic peptidyl prolyl *cis/trans* isomerase (PPIase) activity *in vitro*. The middle domain, which is homologous to other known parvulin-type PPIases, was alone sufficient for this enzymatic activity. Yet all three domains (the N-terminal, PPIase and C-terminal domain) were essential for the function of PrsA *in vivo* in terms of secretion of α-amylase and cell viability. An insertion mutagenesis further characterized the importance of the N-terminal domain since insertions in this part of the protein totally inactivated or reduced the PrsA activity unlike insertions in the PPIase and C-terminal domains in which the insertions were mostly tolerated.

The PPIase domain of PrsA was modelled by taking advantage of the three-dimensional structure of parvulin-type human PPIase hPar14. In the model the amino acid residues predicted to important for the substrate binding and catalytic activity of parvulin-type PPIases were structurally conserved in PrsA. A site-directed mutagenesis of these important residues indeed reduced or abolished the PPIase activity of PrsA *in vitro*. Yet, the substitution of these residues and several other conserved amino acids in the PPIase domain had hardly any effect on the *in vivo* activity suggesting that the enzymatic peptidyl prolyl isomerization activity is not the only activity of PrsA protein and that its essential role *in vivo* seems to depend on some non-PPIase activity of both the PPIase domain and the flanking N- and C-terminal domains.

Saturation of the secretion machinery sets limits on the translocation and secretion and thus engineering of components of the secretion machinery is necessary for enhanced function in biotechnical applications. Saturation of the secretion machinery was studied using AmyQ α-amylase as a model protein and the role of PrsA as a limiting factor for secretion of AmyQ was studied in this context. The capacity of secretion apparatus was determined to be 10 fg/h/cell at the late logarithmic phase of growth meaning a secretion of 30 AmyQ molecules/second. PrsA deficiency did not decrease the capacity of protein translocation confirming that PrsA was not involved in the translocation event itself. Instead, the rate of signal processing was found to be a limiting factor for the translocation of AmyQ. PrsA was a limiting factor for AmyQ secretion in conditions where the AmyQ was overproduced and there was a low level of PrsA but unexpectedly, also in reversed conditions when there was an excess of PrsA compared to the level of AmyQ.

Efficient posttranslocational folding is necessary when heterologous proteins are produced and secreted into the culture medium in *B. subtilis*. Therefore, the effect of PrsA protein and two other factors affecting the late stages of protein folding, namely the negative net charge of the cell wall and the HtrA-type quality control proteases in the membrane-cell wall matrix were studied in this context. A set of eleven proteins with biotechnological interest was used in these experiments. The secretion of four of these model proteins was dependent on PrsA and overproduction of PrsA enhanced secretion of two of them, α-amylase of *B. stearothermophilus* (4-fold) and pneumolysin (1.5-fold). Increasing the net negative charge of the cell wall by mutating the *dlt* operon responsible for the D-alanylation of teichoic acids enhanced the secretion of pneumolysin about 1.5-fold. Decreasing the level of HtrA–type quality control proteases caused harmful effects on growth and did not enhance secretion. The pertussis toxin subunit S1 was found to be a substrate for HtrA-type proteases and its secretion was dependent on these proteases. Results indicate that when components involved in the posttranslocational folding are engineered, secretion of some heterologous proteins is enhanced.

1 INTRODUCTION

Bacteria of the genus *Bacillus* are Gram-positive aerobic endospore-forming rods. The genus is one of the most diverse and commercially useful groups of micro-organisms. Representatives of the genus are found in soil, air and water. The genus has more than 50 described species (Claus and Fritze 1989) and on the basis of taxonomic criteria it is a very heterogeneous group. The majority of the species are non-pathogenic to humans (de Boer and Diderichsen 1991). The only severe human pathogen is *B. anthracis* causing antrax and. *B. cereus* is a mild pathogen responsible for minor infections e.g. food poisoning. *B. subtilis* is the most characterized species of the genus and among Gram-positive bacteria it is often regarded as the model bacterium at the molecular and biochemical level.

Bacillus has a long history in applied microbiology. Fermentation of soya beans by *B. subtilis* to produce a foodstuff called natto has been exploited in Japan for thousands of years (Hara and Veda 1982) and various *Bacillus* species have been exploited in fermentation of cocoa beans for several centuries (Carr 1983). The capacity of secreting high levels of proteins directly into the growth medium, up to 20-25 g/liter has placed *Bacillus* among the most important industrial enzyme producers (Schallmey *et al.,* 2004). Thermophilic species, such as *B. amyloliquefaciens* and *B. licheniformis* produce a great deal of industrial hydrolytic enzymes such as amylases and proteases for the food and detergent market and make up about 50% of the total enzyme market. Besides enzymes, *Bacillus* is used for the production of antibiotics, fine chemicals including flavour enhancers, food supplements and insecticides (Schallmey *et al.,* 2004). Heterologous secreted recombinant proteins have been successfully produced in *Bacillus* as well, but often the yields of secreted proteins in the culture medium have been disappointingly low suggesting bottlenecks in the secretion pathway (Quax 1997; Bron *et al.,* 1998; Braun *et al.,* 1999; Westers *et al.,* 2004). Over the years, the understanding of the molecular mechanism of secretion has been greatly improved and concepts for engineering the machinery towards improved secretion have been developed.

2 REVIEW OF THE LITERATURE

2.1 General secretory pathway in *Bacillus*

All cells need to target newly synthesized proteins to their site of action. For extracellular proteins this involves transport across one or more membranes. In the bacterium cell the cytoplasmic membrane is the first barrier for translocation. Therefore there is machinery for protein translocation in the cytoplasmic membrane to ensure the proper delivery of exoproteins. In Gram-positive bacteria proteins need to pass only one membrane before their release into the external environment. In Gram-negative bacteria passage though the cytoplasmic membrane locates the proteins into the periplasmic space from which proteins need an additional transport mechanism to be translocated into the external environment. The major route for protein translocation across the cytoplasmic membrane in bacteria is the general secretory pathway, also called the Sec-pathway. The Sec-dependent secretion system in the cytoplasmic membrane involves common components both in Gram-positive and in Gram-negative bacteria and it is generally believed that proteins are secreted by similar mechanisms in both groups (van Wely *et al.*, 2001). Sec-dependent system of Gram-positive bacteria is best-characterized in *B. subtilis* (Figure 1). In addition to the general secretory pathway, other protein export systems have been identified in *B. subtilis*: the Tat pathway, (Jongbloed *et al.*, 2000; 2002; van Dijl *et al.*, 2002a), polypeptide translocation by ABC transporters (Havarstein *et al.*, 1995; Paik *et al.*, 1998; Zheng *et al.*, 1999), the pseudopilin pathway involved in natural competence development (Chung and Dubnau 1995; Chung *et al.*, 1998), and phage-like holins (Wang *et al.*, 2000) are specific export systems involved in the transport of a small number of proteins.

To ensure correct targeting of proteins across the membrane, secretory proteins contain a signal peptide that is cleaved by a signal peptidase (SP) during or shortly after translocation (Tjalsma *et al.*, 2000). There are two types of signal peptides in the general secretion pathway of *B. subtilis*: the general signal peptides (type I) and the lipoprotein signal peptides (type II). In the extensive genome-based survey of *B. subtilis* 166 proteins were predicted to contain the type I signal peptide (Tjalsma *et al.*, 2000). Type I signal peptides of *B. subtilis* are 19 to 44 amino acid residues long with the average length of 28 residues. In general, *B. subtilis* signal peptides are five to seven amino acids longer than in *E. coli* (von Heijne 1989). Although the primary structures of different signal peptides show little similarity, three distinct domains can be recognized in these structures: the amino-terminal (N-domain), hydrophobic (H-domain) and carboxyl-terminal (C-domain) regions. The N-domain is rich in

positively charged amino acids containing at least two to three basic residues. The H-domain has an average length of 18 residues and contains hydrophobic residues that adopt α-helical structure. The C-domain has the consensus sequence Ala-Xaa-Ala at position –3 to –1, which is the signal peptidase I cleavage site, and must also adopt an extended β-sheet structure for efficient interaction with the SP (Tjalsma *et al.,* 2000). Lipoproteins have type II signal peptides, which are shorter in length and contain a so-called lipobox with the consensus sequence Leu-(Ala/Ser)-(Ala/Gly)-Cys (Sutcliffe and Harrington 2002) in which the cysteine is the target for lipid modification (see section 2.4.2). Lipoprotein signal peptides are cleaved by signal peptidase II. The number of lipoproteins in *B. subtilis* was predicted to be 114 (Tjalsma *et al.,* 2000). In total 25% of the *B. subtilis* proteome (approximately 300 proteins) have a signal peptide and have the potential to be exported from the cytoplasm across the membrane, most of them via the general secretory pathway. The majority of the proteins are predicted to be membrane-retained either as transmembrane or as lipid-modified proteins, a small percentage of proteins are specifically cell wall-retained. Only 4% of exported proteins are missing a putative retention signal and are released into the external environment (van Dijl *et al.,* 2002b).

2.1.1 Components of the secretion machinery

Most components of bacterial Sec translocation machinery were originally identified by genetic studies in *E. coli* (Bieker *et al.,* 1990; Schatz and Beckwith 1990). The translocation machinery contains cytoplasmic chaperones or targeting factors, a translocation motor, components of the translocation channel and accessory proteins, signal peptidases and proteins involved in the posttranslocational folding (Figure 1). Many of these components are conserved in eubacteria, archaea and eukaryotes (de Keyzer *et al.,* 2003).

Prior to translocation precursor proteins need to maintain an unfolded or loosely folded conformation to be exported through the translocation channel. Molecular chaperones participate in maintaining the precursors in the translocation-competent state (Kusukawa *et al.,* 1989; Wild *et al.,* 1996). A first step in the translocation of preproteins is their targeting to the translocase. This targeting is mediated by specific chaperones and the signal recognition particle (SRP). *E. coli* has a secretion specific chaperone SecB, which interacts with nascent proteins and transfers the precursors to the SecA ATPase of the translocase complex (Randall and Hardy 2002). *B. subtilis* is lacking SecB but contains CsaA chaperone that shows activity reminiscent of *E. coli* SecB. Accordingly, CsaA interacts both with a precursor

protein and SecA, and suppresses the growth and secretion defects of several chaperone mutations in *E. coli* (Muller *et al.,* 2000a; 2000b). Yet there is no sequence similarity between CsaA and SecB.

The best-characterized translocation targeting factor in *B. subtilis* is Ffh GTPase, which is homologous to the main subunit of eukaryotic SRP (Honda *et al.,* 1993; Nakamura *et al.,* 1994). Ffh functions as a general targeting factor for secretory proteins (Bunai *et al.,* 1999; van Wely *et al.,* 2001). It forms a ribonucleoprotein complex with the cytoplasmic RNA and Hbsu histone-like protein (Struck *et al.,* 1989; Nakamura *et al.,* 1999; Yamazaki *et al.,* 1999) and binds to signal peptides emerging on a ribosome followed by targeting the SRP-nascent chain-ribosome complex to the membrane via a specific SRP receptor FtsY (Luirink and Sinning 2004).

SecA is a precursor-stimulated membrane-associated ATPase functioning as the molecular motor to drive the protein translocation (de Keyzer *et al.,* 2003). Studies with *secA* temperature-sensitive mutants show that about 90% of all exported proteins of *B. subtilis* depend on SecA (Hirose *et al.,* 2000). SecA is a homodimeric protein, which contains two nucleotide-binding domains: a high affinity-binding site important for SecA activity and a low affinity-binding site that functions as an intramolecular regulator of ATP hydrolysis (Sianidis *et al.,* 2001). SecA binds to a precursor protein, both to the signal peptide and mature part, and to the SecYEG translocon complex in the membrane. The molecular mechanism of SecA action has been studied in detail (Vrontou and Economou 2004). SecA drives the translocation in a stepwise fashion concomitant with its conformational changes between membrane-inserted and deinserted conformation in a reaction cycle coupled with ATPase activity (van Wely *et al.,* 2001; de Keyzer *et al.,* 2003). SecA has species specificity since *B. subtilis* and *E. coli* SecA are only partially exchangeable (Klose *et al.,* 1993; van der Wolk *et al.,* 1995).

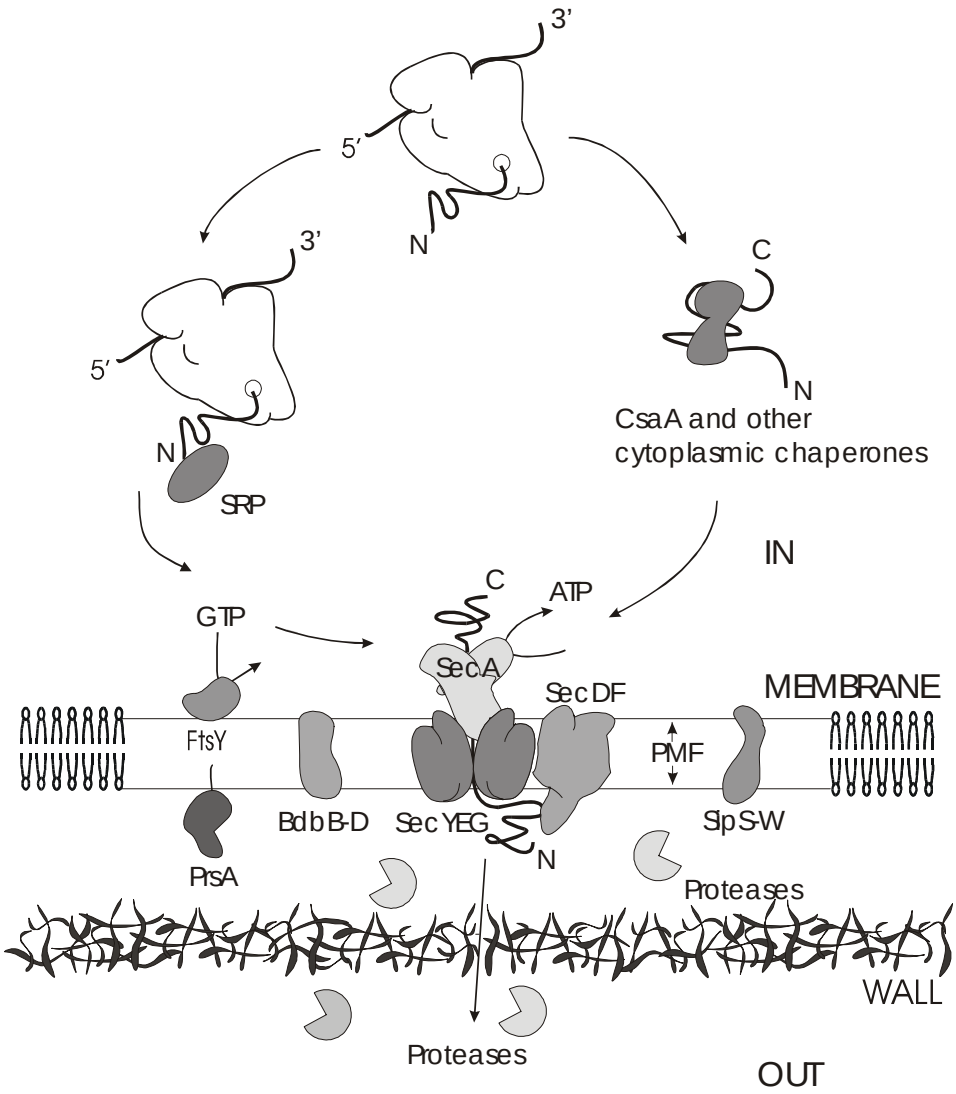


Figure 1. Schematic overview of the general secretory pathway of B. subtilis. Proteins to be translocated across the cytoplasmic membrane are synthesised with an N-terminal signal peptide. In the cytoplasm precursor proteins become associated with chaperones (CsaA) or the signal recognition particle (SRP), which target the precursor proteins to the SecA ATPase. SecA then targets the precursor proteins into the translocase complex, which is composed of the translocation channel (SecYEG) and accessory proteins (SecDF). The signal peptide is removed by signal peptidase (SipS-W).

The subsequent folding of the protein into functionally active protein requires extracytoplasmic folding factors (PrsA and BdbB-D). Proteases reside both at the membrane-cell wall interface and in the external medium. Adapted from van Wely et al., (2001).

The translocation channel is formed of integral membrane proteins SecY, SecE and SecG (van Wely *et al.,* 2001; de Keyzer *et al.,* 2003). The largest subunit in the translocase complex, SecY, (Nakamura *et al.,* 1990b; Nakamura *et al.,* 1990a) is essential for translocation and viability (Breitling *et al.,* 1994). Together with SecE, SecY forms the core of the protein-conducting channel. Similar to SecA, SecY from *E. coli* and *B. subtilis* can complement each other only partially (Nakamura *et al.,* 1990a; Swaving *et al.,* 1999). The second component of the translocon, SecE is also essential for translocation and viability. The SecE of *B. subtilis* and other Gram-positive bacteria are only about half the size of *E. coli* SecE and homologous to the C-terminal part of the *E. coli* SecE (van Wely *et al.,* 2001). SecE protein is exchangeable between species; *B. subtilis* and *Staphylococcus carnosus* SecE complement *E. coli secE* mutants (Meens *et al.,* 1994; Murphy and Beckwith 1994). In association with SecY SecE prevents SecY from being degraded by the FtsH protease (Kihara *et al.,* 1995; Akiyama *et al.,* 1996). However, the stabilization is not the only function but SecE might also contribute to the specificity and catalytic activity of the secretion machinery. The third component of the translocase channel, SecG, is not essential for viability or translocation (van Wely *et al.,* 1999). Yet SecG is not readily functionally exchangeable between *B. subtilis* and *E. coli* (Swaving *et al.,* 1999; van Wely *et al.,* 1999).

SecD and SecF are accessory proteins in the translocation. It is suggested that they function in controlling the catalytic cycle of SecA and maintaining the proton motive force (PMF) (Arkowitz and Wickner 1994; Duong and Wickner 1997). SecD and SecF form a complex with YajC membrane protein and this complex then associates with the SecYEG translocase (Duong and Wickner 1997). Interestingly, in *B. subtilis* SecD and SecF are fused to a single membrane protein (Bolhuis *et al.,* 1998). Like SecG, SecDF is unable to complement the corresponding mutation in *E. coli* though the depletion of *secDF* does not cause any severe defect in translocation or viability (van Wely *et al.,* 1999). An additional protein associated with SecYEG in *E. coli* is YidC that is involved in the insertion of hydrophobic sequences of proteins into the membrane (Scotti *et al.,* 2000). *B. subtilis* has two YidC homologs SpoIIIJ and YqjG (van Wely *et al.,* 2001).

Besides the Sec components anionic phospholipids are essential for protein translocation and SecA activity (van der Does *et al.,* 2000). An increase in the amount of anionic phospholipids was shown to restore protein translocation in *secA*

and *secG* mutants (Suzuki *et al.*, 1999). The energy for translocation is provided by hydrolysis of ATP by SecA and PMF (de Keyzer *et al.*, 2003). ATP is essential for the initiation of translocation and after SecA is no longer associated with the SecYEG translocase PMF can further drive the reaction (Schiebel *et al.*, 1991).

Processing of precursor proteins is a prerequisite for the release of exported proteins from the membrane. There are five type I signal peptidases (SPs), SipS-W, in *B. subtilis*, and some strain have a sixth one, SipP, encoded by a plasmid (Meijer *et al.*, 1995; Tjalsma *et al.*, 1998). Five of the SPs (SipP, S, T, U and V) closely resemble each other in possessing an N-terminal membrane anchor and thus belonging to the P-type class of SPs found in eubacteria. SipW differs from the other SPs since it has an additional C-terminal anchor and belongs to the family of ER-type signal peptidases found in archaea and in the endoplasmic reticulum (ER) of eukaryotes (Tjalsma *et al.*, 1998). There is functional redundancy among SPs as well as differences in specificity (Bron *et al.*, 1998). Cells in which up to four SPs are depleted are viable, only the deletion of both *sipS* and *sipT* is lethal (Tjalsma *et al.*, 1997). *B. subtilis* contains only one type II signal peptidase Lsp (Pragai *et al.*, 1997). However, the *lsp* null mutant is viable and some mature form of a lipoprotein appear in the mutant indicating some alternative processing of lipoproteins in the absence of Lsp (Leskelä *et al.*, 1999a; Tjalsma *et al.*, 1999).

2.2 Protein folding in the translocation pathway

To become an active protein a newly synthesized peptide chains must fold into a correct three-dimensional (3D) conformation. Some small single-domain proteins can fold correctly spontaneously (Dobson and Karplus 1999). In contrast, the folding of larger proteins involves partially folded intermediates including misfolded ones that tend to aggregate. Therefore, these proteins need assistance in folding.

Protein folding is crucial in the translocation pathway. Firstly, precursors need to maintain the unfolded or loosely folded conformation prior to the translocation. This is accomplished by cytosolic chaperones (Table 1). These chaperones hold the precursor proteins in the unfolded conformation, dissolve aggregated proteins, and co-operate with proteases when the aggregated proteins need to be degraded (Dougan *et al.*, 2002). Secondly, after the translocation across the cytoplasmic membrane, the proteins need to fold correctly. To overcome this problem, there are spesific proteins on the *trans* side of the cytoplasmic membrane to assist the folding. These proteins include extracytoplasmic chaperones, proteins involved in disulphide bond formation and isomerization, and peptidyl prolyl *cis/trans* isomerases (Table 1).

Table 1. Proteins involved in protein folding in the translocation pathway of bacteria

Trigger factor	Ribosome-bound chaperone and PPIase
DnaK	Hsp70 chaperone stabilizes hydrophobic regions in proteins
DnaJ	Hsp40 co-chaperone of DnaK
GrpE	Nucleotide exchange factor of DnaK
GroEL	Hsp60 chaperone forms a cylinder with a central hole in which synthesized proteins are protected from aggregation and are able to fold
GroES	Hsp10 co-chaperone of GroEL
HtpG	Hsp90 chaperone, role unclear in bacteria
ClpB subfamily	Hsp100 chaperones with protein disaggregating activity used in co-operation with DnaK
ClpA subfamily	Hsp100 chaperones with unfolding activity used in co-operation with proteases
Small Hsps	Chaperones bind to aggregation-prone proteins and maintain them in the refoldable form, found in inclusion bodies and intracellular aggregates
SecB	Secretion specific chaperone
CsaA	Secretion specific chaperone in *B. subtilis*, function similar to SecB
Periplasmic chaperones	Fold e.g. outer membrane proteins and proteins of adhesive organelles in the periplasmic space
Dsb/Bdb	Periplasmic or membrane-bound proteins catalyzing formation and isomerization of disulphide bonds
PPIases	Proteins found in all cell compartments catalyzing isomerization of the peptidyl prolyl bond

Protein nomenclature is according to *E. coli* and *B. subtilis.*

2.2.1 Molecular chaperones

The classical definition for chaperones is that they are proteins that protect and prevent nascent proteins from misfolding and aggregation but do not contribute any conformational information e.g. enzymatic activity in the folding process itself (Hartl and Hayer-Hartl 2002). Nowadays, the definition of chaperones is often used in a more general content when discussing protein folding. In general, chaperones recognize hydrophobic residues and unstructured regions that are present in unfolded or misfolded proteins but are not present upon complete folding (Dougan *et al.*, 2002). Many chaperones are constitutively expressed but their expression is increased under stress conditions such as high temperature, and therefore they are classified as stress proteins or heat shock proteins (Hsps) (Gething and Sambrook 1992).

2.2.1.1 Cytosolic chaperones

Cytosolic chaperones maintain the precursor proteins in their translocation-competent conformations prior to the translocation. According to Dougan *et al.,* (2002), these chaperones can be categorized into three groups on the basis of their mode of action: folders (e.g. DnaK and GroEL) refold the misfolded or aggregated substrates, holders (e.g. small Hsps) prevent the aggregation by binding to the aggregation-prone substrates but are unable to refold the protein, and unfolders (e.g. Cpls), which primarily unfold the proteins in preparation for subsequent degradation. Together chaperones constitute a network of proteins involved in not only in general protein quality control but also in regulation and in the management of specific protein-folding pathways (Dougan *et al.*, 2002).

The first chaperone to interact with a nascent polypeptide is trigger factor (TF). TF is a eubacterial protein first identified in *E. coli* as a factor triggering the translocation of Omp proteins into membrane vesicles in cell free systems (Crooke and Wickner 1987). TF is located on the large subunit of ribosome next to the peptide exit channel where it binds to the nascent peptide chains and assists the cotranslocational folding. TF exhibits a chaperone function as well as a peptidyl prolyl *cis/trans* isomerase (PPIase) activity (see sections 2.2.3.2 and 2.2.3.4). The protein is dispensable for viability. The combined deletion of *tig* encoding TF and *dnaK* causes aggregation of proteins and lethality indicating overlapping functions of TF with the Hsp70 chaperone system (Deuerling *et al.*, 1999). However, the *tig dnaK* double mutant is viable under specific growth conditions suggesting yet another chaperone system with overlapping function in the double mutant (Reyes and Yoshikawa 2002; Genevaux *et al.*, 2004).

The Hsp70 (DnaK) family of chaperones function together with the co-chaperones of Hsp40 (DnaJ) family in an ATP-dependent manner by binding to polypeptides and stabilizing their hydrophobic regions in protein folding (Bukau and Horwich 1998). In bacteria the Hsp70 system is the most abundant chaperone systems involving DnaK, DnaJ and the nucleotide exchange factor GrpE. During the Hsp70 assisted folding substrates undergo repeated cycles of binding and release reaction with the chaperone complex. In *B. subtilis* both *dnaK* and the *groES* operon are regulated by the HrcA repressor. This repressor binds to the sequence of tandem repeats designated CIRCE (controlling inverted repeat of chaperone expression) in the promotor area and regulates the expression of the genes belonging to the CIRCE regulon (Zuber and Schumann 1994; Mogk *et al.*, 1997).

Chaperonins are large barrel-like complexes consisting of two stacked rings forming a cylinder with a central hole in which a polypeptide can fold in a unique

environment. Chaperonin GroEL (also called Hsp60) is the best-characterized chaperone, and the *E. coli* GroEL has become the model for chaperone function. GroEL is highly conserved in bacteria and essential for viability (Walter 2002). GroEL-assisted protein folding is a three-step process: capture, folding and release (Ranson *et al.*, 1997). During the reaction cycle the co-chaperone GroES and ATP bind to GroEL and several cycles occur until the polypeptide is folded and is no longer recognized by GroEL. The size restriction for GroEL-mediated folding seems to be ~ 60 kDa, larger proteins can bind to GroEL but do not fit into the GroEL cylinder (Ewalt *et al.*, 1997; Sakikawa *et al.*, 1999). As much as 40% of *E. coli* proteins can bind to GroEL (Viitanen *et al.*, 1992) but it is unclear how many of them are stringently dependent on GroEL for their folding (Walter 2002).

Hsp90 is an abundant and highly conserved molecular chaperone essential in eukaryotes found in several cell compartments in several isoforms (Picard 2002). In contrast, in prokaryotes Hsp90 has an auxiliary role. Little is known about the bacterial Hsp90 homologue HtpG even though this chaperone is highly expressed upon heat shock. In *E. coli* strains devoid of HtpG behave like wild type (wt) strains and show no specific phenotypes (Bardwell and Craig 1987). In *B. subtilis*, *htpG* belongs to the class IV group of heat shock genes and is induced more by absolute temperature rather than by temperature increase. Moreover, the appearance of nonnative proteins in the cytoplasm does not enhance transcription of *htpG* (Versteeg *et al.*, 2003). The current understanding of Hsp90 thus comes from eukaryotes, mainly from the *in vitro* studies of maturation of steroid receptors (Pratt and Toft 2003). An interesting feature of Hsp90 is that its function and substrate recognition are modulated by a large variety of co-chaperones e.g. PPIases (section 2.2.3) serve as co-chaperones in the Hsp90 complexes (Prodromou *et al.*, 1999; Picard 2002).

Small Hsps (sHsps) are a quite unknown family of small heat shock proteins (15-40 kDa) (Plesofsky-Vig *et al.*, 1992). Only a few sHsps have been studied in detail, and therefore the function of sHsps is unclear. A common feature in sHsps is the C-terminal so-called α-crystallin domain and the organization into large oligomeric structures (Haslbeck *et al.*, 1999; van Montfort *et al.*, 2001). The main role of sHsps in the chaperone network seems to be efficient binding to the aggregation-prone proteins and maintaining them in a refoldable form. Indeed two *E. coli* sHsps (IbpA and IpbB) were found to be associated with intracellular protein aggregates and with inclusion bodies in protein overproduction conditions (Allen *et al.*, 1992). In cooperation with the Hsp70/DnaK system the sHsp-bound proteins can be released and refolded into their native states (Veinger *et al.*, 1998; Haslbeck 2002).

The Hsp100/Clp family of chaperones dissolve protein aggregates and disassemble protein structures (Maurizi and Di 2004; Weibezahn *et al.*, 2004). These proteins are divided into two subfamilies with distinct enzymatic functions. The ClpB subfamily displays protein disaggregating activity that is used in co-operation with Hsp70/DnaK systems (Ben-Zvi and Goloubinoff 2001) whereas members of the ClpA subfamily have unfolding activity and act primarily in cooperation with proteases, such as ClpP and ClpQ, to catalyze proteolysis (Horwich *et al.*, 1999; Weibezahn *et al.*, 2004) but may act as molecular chaperone independently of the proteases as well (Wickner *et al.*, 1994; Wawrzynow *et al.*, 1996). Hsp100 proteins belong to the AAA^+ superfamily (ATPase associated proteins with various cellular activities), which is a ubiquitous family of ATP-dependent proteins (Schirmer *et al.*, 1996; Maurizi and Di 2004). Morever, Hsp100 proteins assemble into hexameric or heptameric rings (Beuron *et al.*, 1998; Ortega *et al.*, 2000; Sousa *et al.*, 2000) reminiscent to GroEL but exhibit no sequence similarity to chaperonins (Schirmer *et al.*, 1996; Horwich *et al.*, 1999). In *B. subtilis* the Clp proteins play essential roles in DNA competence, sporulation, motility and in several stress conditions (Msadek *et al.*, 1994; Slack *et al.*, 1995; Gerth *et al.*, 1996; Msadek *et al.*, 1998). ClpA and ClpB are missing in *B. subtilis* but instead there is the ClpC protein that functionally resembles both ClpA and ClpB (Turgay *et al.*, 1997). Most Clp genes of *B. subtilis* belong to the class III group of heat shock genes and are controlled by the CtsR DNA-binding protein (Derre *et al.*, 1999).

2.2.1.2 Extracytosolic chaperones

Once proteins have been translocated across the cytoplasmic membrane they need to fold into their active conformation. Correct folding is crucial to obtain biological activity and to avoid proteolytic degradation. To overcome the problem of folding, there are proteins on the *trans* side of the cytoplasmic membrane to assist the folding. In Gram-negative bacteria the periplasmic space is the cell compartment into which proteins enter after the translocation. The knowledge of periplasmic chaperones comes mainly from studies of PapD-like chaperones (Behrens 2003). These highly conserved and specialized chaperones direct the assembly and folding of several adhesive organelles. (Knight *et al.*, 2000). The PapD-like proteins function as *trans*-acting steric chaperones that direct folding of proteins by supplying essential steric information (Barnhart *et al.*, 2000). In addition to PapD-like proteins, other periplasmic chaperones such as Skp for outer membrane proteins and Lol for lipoproteins have been identified (Miyamoto *et al.*, 2002; Bulieris *et al.*, 2003). In addition to chaperones, thiol-sulphide oxidoreductases and PPIases in the periplasmic space act in the folding process (see sections 2.2.2 and 2.2.3).

Less is known about extracytoplasmic chaperones in Gram-positive bacteria. *B. subtilis* contains PrsA lipoprotein, which is homologous to PPIases and serves as an essential folding factor for some exoproteins (see section 2.2.4). In addition, several membrane-associated thiol-disulphide oxidoreductases have been identified (see section 2.2.2). In *B. subtilis*, divalent cations and components of cell wall function as folding factors as well (Sarvas *et al.*, 2004).

2.2.2 Thiol-disulphide oxidoreductases

One of the key steps in the protein folding is the formation of disulphide bonds between cysteine residues (Hiniker and Bardwell 2003). Generally, disulphide bonds are found in proteins exported outside the cytoplasm. In eukaryotes, the bond formation occurs in the ER. In Gram-negative bacteria the bond formation takes place in the periplasmic space in which the proteins will reside or they will be secreted. In contrast, less is known about disulphide bond formation in Gram-positive bacteria. Yet the bond formation does take place on the *trans* side of the cytoplasmic membrane (Bolhuis *et al.*, 1999a; Tjalsma *et al.*, 2000). The fact that secreted eukaryotic proteins contain more disulphide bonds than bacterial proteins hinder in many cases their production in bacterial hosts since incorrect disulphide bond formation or lack of disulphide bonds leads to nonnative product and poor secretion (Saunders *et al.*, 1987; Bolhuis *et al.*, 1999a).

In eukaryotes, a single protein, disulphide isomerase (PDI) located in the ER, is capable of catalysing both the formation and the isomerization of disulphide bonds. However, in prokaryotes there are several proteins involved in the process. In *E. coli*, Dsb thiol-disulphide oxidoreductases are responsible for catalyzing disulphide bond reactions. These proteins belong to the thioredoxin superfamily characterized by thioredoxin fold and the Cys-Xaa-Xaa-Cys motif responsible for redox function (Raina and Missiakas 1997; Fomenko and Gladyshev 2003). There are two pathways in *E. coli*; the DsbA-DsbB pathway oxidizes thiol groups to form disulphides while the DsbC-DsbD pathway isomerizes incorrect disulphide pairs (Hiniker and Bardwell 2003). DsbA introduces disulphide bonds into newly secreted proteins by interacting with proteins containing reduced cysteines and oxidizing them. DsbA has a strong oxidation power due to the unusually low pK_a of the N-terminal cysteine residue in the Cys-Xaa-Xaa-Cys motif (Collet and Bardwell 2002). In order to DsbA to regain the oxidized, more stable form, the inner membrane protein DsbB reoxidizes it and in turn donates the electrons to ubiquinone and finally to molecular oxygen in aerobic conditions or to menaquinone and anaerobic electron acceptors in anaerobic conditions (Hiniker and Bardwell 2003).

DsbC catalyses isomerization of disulphide bonds of proteins containing multiple disulphide bonds; so far identified *in vivo* substrates are RNase I and endopeptidase MepA. In addition to the isomerase activity, DsbC possesses a peptide-binding activity that enhances the interaction with proteins and a chaperone activity; it can assist the refolding of model proteins *in vitro* (Chen *et al.,* 1999). *E. coli* has an additional disulphide isomerase, DsbG with 56% sequence similarity to DsbC (Andersen *et al.,* 1997). DsbG has a chaperone activity as well; it prevents aggregation of model proteins *in vitro* and this activity is independent of its disulphide redox state (Shao *et al.,* 2000). DsbG also complements *dsbC* mutation in the refolding of some eukaryotic proteins in *E. coli*. Yet, no *in vivo* substrate of DsbG has been discovered (Hiniker and Bardwell 2004).

Both DsbC and DsbG need to remain in a reduced state to act as isomerases in a highly oxidizing environment. The reduced state of these proteins is maintained by the action of the inner membrane protein DsbD (Missiakas *et al.,* 1995; Goldstone *et al.,* 2001). The ultimate source of reducing potential is NADPH that donates electrons to thioredoxin that in turn reduces DsbD (Krupp *et al.,* 2001). DsbD is composed of three domains and each domain has a pair of cysteine residues that participate in disulphide exchange reactions and sequentially transfer the electrons (Katzen and Beckwith 2000). In addition to maintaining the isomerization activity of DsbC and DsbG, DsbD plays an essential role in cytochrome biosynthesis and is important in copper resistance (Crooke and Cole 1995; Fong *et al.,* 1995).

In many cases the secretion of heterologous proteins containing disulphide bonds is insufficient in *Bacillus.* Yet some proteins such as human interleukin-3 and *E. coli* alkaline phosphatase are secreted efficiently in an active form indicating the presence of functional oxidoreductases on the outer surface of the cytoplasmic membrane (Bolhuis *et al.,* 1999a; Tjalsma *et al.,* 2000). Database searches revealed four putative homologs of *E. coli* Dsb proteins in *B. subtilis* and these protein were designated Bdb (*Bacillus* disulphide bond) (Bolhuis *et al.,* 1999a; Meima *et al.,* 2002). BdbA shares similarity with DsbA while BdbB and BdbC are similar to DsbB. The fourth Bdb protein, BdbD, is similar to the DsbA of *S. aureus* and DsbG of *E. coli* and other Gram-negative bacteria. Knock-out mutant analyses revealed that unlike Dsb protein in *E. coli*, none of the four *bdb* genes is essential for growth, viability or resistance to reducing agents (Bolhuis *et al.,* 1999a; Meima *et al.,* 2002). However, a model protein *E. coli* alkaline phosphatase PhoA containing two disulphide bonds was unstable in *bdbB*, *bdbC* and *bdbD* mutants and its secretion was decreased. Cells lacking *bdbC* also showed decreased stability TEM-1 β-lactamase, which is another model protein with disulphide bonds. BdbA was not required for the stability of PhoA or TEM-1 β-lactamase. Data indicate that BdbB, BdbC and BdbD have a general role in disulphide formation whereas BdbA has a

more specific function. In addition to the secretion defect of model proteins, there was a reduced amount of competence protein ComGC in cells lacking *bdbC* or *bdbD*, and the cells were unable to develop competence for DNA uptake. Results indicate that BdbC and BdbD catalyse the formation of disulphide bonds that are essential for the DNA binding and uptake machinery. Presumably BdbC and BdbD functionally correspond to *E. coli* redox pair DsbA and DsbB (Erlendsson and Hederstedt 2002; Meima *et al.*, 2002).

2.2.3 Peptidyl prolyl *cis/trans* isomerases

Cis/trans isomerization of prolyl bonds is a slow step in protein folding and often limits the rate of folding (Schmid *et al.*, 1993). Peptide bond in the polypeptide chain is planar and therefore the bond can be either in *trans* or *cis* conformation. Most of the peptide bonds synthesised are in the *trans* form and this is also the case in the native proteins. The *trans* conformation is especially strongly favoured in proteins that do not contain proline residues due to high energy barrier, the *cis* content of nonprolyl peptide bonds is only 0.1-0.5% (Reimer *et al.*, 1998). However, peptide bonds that precede a proline residue (Xaa-Pro) are more often found in the *cis* form (Figure 2). In these prolyl bonds the required free energy between the two conformations is less and equilibrium of both forms occur. Still, *trans* is the dominant form (60-90%) a smaller portion of prolyl bonds being in *cis* (10-40%) (Schmid 2001). There are specific enzymes, peptidyl prolyl *cis/trans* isomerases (PPIases) that catalyse this isomerization reaction of prolyl bonds. The actual *cis/trans* ratio depends on the size and chemical nature of the residue preceding proline as well as the flanking amino acids (Reimer et al., 1998). *Cis/trans* isomerization is in any case a slow reaction with time constants of 10 to hundred seconds at 25°C (Schmid 2001).

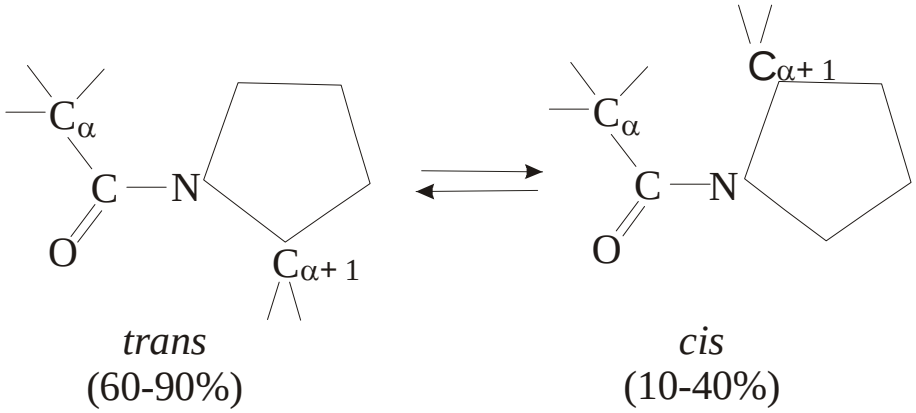


Figure 2. Isomerization between the cis and trans forms of a Xaa-Pro peptide bond. Xaa is any amino acid. Adapted from Schmid et al., (2001).

There are several assays for measurement of the PPIase activity *in vitro*. The protease-coupled assay exploits the conformational specificity of chymotrypsin that cleaves a chromogenic reporter group from a substrate tetrapeptide Ala-Xaa-Pro-Phe 4-nitroanilide only then the Xaa-Pro bound is in the *trans* conformation creating a yellowish colour detected spectrofotometrically (Fischer *et al.,* 1984). In aqueous solution at equilibrium, 90% of tetrapeptides contain *trans* bond and these are hydrolysed rapidly in the presence of chymotrypsin. The remaining 10% are cleaved slowly due to the limiting rate of *cis-trans* isomerization of the Xaa-Pro bond. The slow hydrolysis is accelerated in the presence of a PPIase. This assay was improved by increasing the fraction of the *cis*-isomer by dissolving the tetrapeptide in an anhydrous solvent (Kofron *et al.,* 1991). However, the use of chymotrypsin protease generates problems with proteolysis-prone PPIases. To overcome the protease problem, an uncoupled protease-free assay based on different coefficients for the *cis* and *trans* conformation of tetrapeptide substrates was developed (Janowski *et al.,* 1997).

Ribonuclease T1 (RNase T1) is often used as a substrate protein when determining the refolding activity of a PPIase (Schmid *et al.,* 1996). The folding mechanism of RNase T1 is thoroughly studied making it an excellent model protein (Mayr and Schmid 1993; Schmid *et al.,* 1996; Schmid 2001). RNase T1 is a small single-domain protein with four prolines and two disulphide bonds (Martinez-Oyanedel *et al.,* 1991). Two of the proline residues, Pro39 and Pro55, are in the *cis*-form. Catalysis of RNase T1 refolding by PPIases is most efficient in the absence of disulphide bonds. However, the oxidized form of RNase T1 with intact disulphide bonds can also be used (Ramm and Pluckthun 2001) but in the presence of intact

disulphide bonds the protein is more stable and partially folded intermediate forms rapidly disabling some PPIases to catalyse the refolding. Therefore the refolding activity may be lower compared to the reduced form of RNase T1 devoid of disulphide bonds (Göthel and Marahiel 1999).

There are three families of PPIases: cyclophilins, FK506-binding proteins and parvulins. Cyclophilins and FK505-binding proteins are often called immunophilins due to their binding to immunosuppressant compounds that are used as an immunosuppressive treatment in organ and tissue transplantations to provide prophylaxis and prevent allograft rejection (Galat 2003). All three classes of PPIases are ubiquitous proteins found in all kingdoms of life and are expressed in many tissues and cell compartments. PPIases are either single-domain proteins capable of PPIase activity and inhibitor binding only, or the PPIase domain is part of a larger protein with additional domains and functions. Only a few PPIases are essential and in most cases the exact functional role of individual PPIases is unknown (Schmid 2001).

2.2.3.1 Cyclophilins

Cyclophilins (Cyps) are a family of PPIases inhibited by cyclosporin A (CsA) produced by many fungi imperfecti (Borel 1989). The best-characterized Cyp is the mammalian Cyp18 (hCyp18), which is a cytosolic single-domain protein expressed abundantly in all tissues. It has a high CsA sensitivity and high efficiency for tetrapeptide isomerization. hCyp18 is a specially interesting PPIase since it is a potential drug target for anti-HIV therapy; the interaction between hCypA and HIV-1 gap protein is required to promote the assembly of the viral core (Colgan *et al.*, 1996). Several mammalian Cyp isoforms differing in their subcellular location and binding affinity to CsA have been identified and they all share a sequence similarity of over 50% with hCyp18 (Göthel and Marahiel 1999). In general, Cyps are structurally highly conserved. Structure analysis of hCyp18 and *E. coli* Cyp demonstrate that Cyps fold into a β-barrel in which eight β-strands are capped at both ends by α-helices (Kallen and Walkinshaw 1992; Clubb *et al.*, 1993; 1994). Multi-domain Cyps contain a domain homologous with hCyp18 and additional domains. Cyp40 is a two-domain cyclophilin with N-terminal PPIase domain and a C-terminal tetratricopeptide repeat (TPR) domain (Kieffer *et al.*, 1993). Cyp40 is associated by the TPR domain to Hsp90 in the steroid complex and is involved in the maturation of the complex harbouring both a PPIase activity and chaperone activity (Bose *et al.*, 1996).

Cyclophilins have been identified in genomes of various prokaryotes but a few of them have been characterized any further. *E. coli* has two cyclophilins, a periplasmic

PpiA and its cytosolic counterpartner (Hayano *et al.*, 1991). *B. subtilis* has a single cyclophilin, PpiB that together with the trigger factor are the only cytosolic PPIases known in *B. subtilis* (Herrler *et al.*, 1994; Göthel *et al.*, 1998). PpiB has a moderate CsA affinity (Achenbach *et al.*, 1997) and it catalyses tetrapeptide isomerization with low efficiency (1.1×10^3 M^{-1} s^{-1}) as well as refolding of RNase T1 (3.8×10^4 M^{-1} s^{-1}) (Göthel *et al.*, 1996; 1998). PpiB is non-essential in a rich medium and under various stress conditions, only in the starvation situation the double mutant of *ppiB* and *tig* encoding trigger factor showed retarded growth indicating that PPIases become essential in these conditions (Göthel *et al.*, 1998).

2.2.3.2 FK506-binding proteins

FK506-binding proteins (FKBPs) are a family of PPIases inhibited by immunosuppressive drugs FK506 or rapamycin produced by *Streptococcus* species (Göthel and Marahiel 1999). The best-characterized FKBP is FKBP12, a 12 kDa cytosolic single-domain protein. FKBP12 is composed of five-stranded antiparallel β sheets wrapped around a short α-helix linked together with flexible loops (Van Duyne *et al.*, 1993). Small FKBPs like FKBP12 modulate signal transduction pathway by binding to several receptors (Schmid 2001). Best-characterized multidomain FKBPs, FKBP51 and FKBP52 contain a FKBP12-like domain, a TPR-domain and a C-terminal domain, and like Cyp40, are associated with Hsp90 by their TPR-domain. Consistent with Cyp40, the large FKBPs are involved in protein folding possessing both a PPIase and chaperone activity *in vitro* (Pirkl and Buchner 2001; Pirkl *et al.*, 2001). The binding of immunophilins to the Hsp90 complex is competitive and preferential depending on a receptor type suggesting regulatory role for the immunophilins in the complex (Picard 2002).

Prokayotic FKBPs have specific known functions. The macrophage infectivity potentiator (Mip) of *Legionella pneumophila* aids bacterial infection of human macrophages, and its homologs are found in various prokaryotic genomes (Bangsborg *et al.*, 1991; Riboldi-Tunnicliffe *et al.*, 2001; Kohler *et al.*, 2003). *E. coli* FkpA is a dimeric periplasmic Mip-homolog participating in the protein folding of periplasmic proteins (Horne and Young 1995; Missiakas *et al.*, 1996). FkpA has been identified to possess an additional chaperone function as well (Arie *et al.*, 2001). Another FKBP of *E. coli* is cytosolic SlyD (Roof *et al.*, 1994; 1997). A known function of SlyD is the stabilization of the lysis E protein needed for host cell lysis in phage-infected cells (Bernhardt *et al.*, 2002).

Trigger factor (TF) is a prokaryotic ribosome-bound protein with the FKBP fold consisting of an N-terminal ribosome-binding domain, a middle FKBP domain and a

C-terminal domain with unknown function (see section 2.2.1.1). The interesting feature of the *E. coli* TF is that it is not inhibited by FK506 (Stoller *et al.*, 1995). Furthermore, the FKBP domain of TF is not essential for the folding of cytosolic proteins in *E. coli* (Kramer *et al.*, 2004). Characteristically, TF catalyzes the RNase T1 refolding 20-100 fold more efficiently (1-1.5 x 10^6 $M^{-1}s^{-1}$) than other PPIases (Stoller *et al.*, 1995; Scholz *et al.*, 1997a; Göthel *et al.*, 1998; Kramer *et al.*, 2004) due to a very tight binding to the substrate but have hardly any activity towards tetrapeptides (Stoller *et al.*, 1995). In *B. subtilis*, TF is the only FKBP identified. It catalyses the tetrapeptide isomerization poorly (0.71 $\mu M^{-1}s^{-1}$) but refolding of RNase T1 very efficiently (1.4 x 10^6 $M^{-1}s^{-1}$). Deletion of *tig* encoding TF is not lethal, only under starvation condition a double deletion of *ppiB* encoding a cyclophilin and *tig* caused retarded growth (Göthel *et al.*, 1998).

2.2.3.3 Parvulins

Parvulins are the third family of PPIases. This family is named after its first member Parvulin of *E. coli* (Rahfeld *et al.*, 1994b; 1994a). Parvulin contains only 92 residues being one of the smallest enzymes known (Schmid 2001). It has a very high PPIase activity toward a tetrapeptide (1.7 x 10^7 $M^{-1}s^{-1}$) (Rahfeld *et al.*, 1994a) and moderate activity for RNase T1 (3 x 10^4 $M^{-1}s^{-1}$) (Scholz *et al.*, 1997a). There is no specific inhibitor for all parvulins but juglone (5-hydroxy-1,4-naphthoquinone) irreversibly inhibits *in vitro* PPIase activity of those parvulins containing a cysteine residue in the active site of the enzyme. Juglone inhibits these enzymes by binding covalently to the side chains of cysteine (Hennig *et al.*, 1998). Specific potential inhibitors for some parvulins have been identified (Uchida *et al.*, 2003). The 3D-structures of parvulins reveal that parvulin-type PPIase domain consists of four antiparallel β-sheets and four α-helices forming a flattered β-barrel surrounded by helices (Ranganathan *et al.*, 1997; Sekerina *et al.*, 2000; Terada *et al.*, 2001; Bitto and McKay 2002; Kuhlewein *et al.*, 2004). Data reveal that although the sequence similarity between parvulins and FKBPs is low, their 3D-structures are similar (Ranganathan *et al.*, 1997; Sekerina *et al.*, 2000).

Human Pin1 is the best-characterized parvulin. Pin1 (139 aa) is an essential nuclear mitotic regulator for the G2/M transition of the eukaryotic cell cycle (Lu *et al.*, 1996). It consists of a WW protein-protein interaction motif and PPIase domain organized around a hydrophobic cavity (Ranganathan *et al.*, 1997). The conserved residues of the substrate-binding pocket (Leu122, Met130 and Phe134) project outward from the β-barrel. The binding pocket is surrounded by the active site residues (Cys113, His59, His157 and Ser154), which are postulated to work as a catalytic cascade of a nucleophilic mechanism in the isomerization reaction (Fischer *et al.*, 1989; Ranganathan *et al.*, 1997). A triad of basic residues (Lys63, Arg68 and

Arg69) resides at the entrance to the active site. This basic cluster is responsible for the strong preference of Pin1 to phosphorylated peptide and protein substrates. Prolyl isomerization activity increased 1300-fold compared to nonphoshorylated substrates when phosphorylated tetrapeptides were used (Ranganathan *et al.,* 1997). Natural substrates of Pin1 are the Ser/Thr-Pro motifs of protein kinases and phosphatases in the cell cycle; Pin1 binds to them in their phosphorylated form and thereby acts as a general regulator of mitotic proteins (Shen *et al.,* 1998). Pin1-type parvulins have been identified in several organisms including Ssp1 of *Neurospora crassa* (Kops *et al.,* 1998), Ess1/Ptf1 of *Saccharomyces cerevisiae* (Hani *et al.,* 1995), Dodo of *Drosophila* (Maleszka *et al.,* 1996) and in plants (Landrieu *et al.,* 2000; Metzner *et al.,* 2001; Yao *et al.,* 2001). These parvulins form a subfamily of Pin1-like phosphate specific parvulins (Sekerina *et al.,* 2000).

A second subfamily consists of human Parvulin hPar14-like parvulins. hPar14 contains a nonstructured N-terminal extension followed by a PPIase domain (Sekerina *et al.,* 2000; Terada *et al.,* 2001). hPar14 has a role in cell cycle and chromatin modeling (Fujiyama *et al.,* 2002; Surmacz *et al.,* 2002). The PPIase domain of hPar14 adopts an identical 3D-structure to Pin1 (Sekerina *et al.,* 2000). However, hPar14 lacks the basic residues responsible for the preference for phosphorylated substrates and instead it prefers positively charged substrates, especially arginine preceding proline (Uchida *et al.,* 1999). Otherwise the substrate-binding pocket of hPar14 is conserved. Of the residues responsible for the prolyl isomerization two histides are conserved but Cys113 is replaced with Asp and Ser154 with Phe. In many bacterial parvulins Asp is also found in place of Cys e.g. PrsA of *B. subtilis*, PrtM of *Lactococcus lactis* and SurA and PpiD of *E. coli* are bacterial parvulins with Asp in their active site (Terada *et al.*, 2001). Additionally, Ser is often replaced by another residue in several parvulins. It seems that structured conservation of the active site can be maintained even with some changes in the amino acid composition and that prolyl isomerization can still occur. Yet the mechanism of catalysis might be differerent with different active site residues (Sekerina *et al.,* 2000; Terada *et al.*, 2001).

The bacterial parvulins form the third subfamily of parvulins. These enzymes are mainly involved in the folding, maturation and stability of proteins (Vos *et al.,* 1989; Jacobs *et al.,* 1993; Rouviere and Gross 1996; Dartigalongue and Raina 1998). PrsA of *B. subtilis* is an essential extracytoplasmic lipoprotein needed for the posttranslocational folding of exoproteins (see section 2.2.4). PrtM of *L. lactis* is required for maturation of cell wall protease PrtP. In *E. coli*, periplasmic PpiD and SurA assist the stability and folding of outer membrane proteins. Interestingly, SurA contains two PPIase domains (P1 and P2) from which the inactive P1 together with N- and C-terminal domains forms a core module while the active P2 is a satellite

domain tethering from the core. The core module has a large crevice for peptide binding possibly needed for the additional chaperone function of SurA (Bitto and McKay 2002).

2.2.3.4 Additional chaperone activity of PPIases

In addition to the enzymatic isomerization activity, some PPIases have a chaperone activity that can reside either in the PPIase domain as a PPIase-dependent activity or in an adjacent domain independent of the PPIase domain. Eukaryotic Hsp90-associated immunophilins are able to refold several substrate proteins even in the presence of PPIase inhibitor indicating PPIase-independent chaperone activity (Bose *et al.,* 1996; Freeman and Morimoto 1996; Pirkl and Buchner 2001; Pirkl *et al.,* 2001). SurA and FkpA also have a PPIase-independent activity; the *surA* mutant lacking both PPIase domains has a chaperone activity *in vitro* (Behrens *et al.,* 2001) and the *fkpA* mutant having the C-terminal PPIase domain but lacking the N-terminal domain was devoid of chaperone activity (Saul *et al.,* 2004). Conversely, in the trigger factor of *E. coli* both the chaperone and PPIase activity involve the same binding pocket in the FKBP domain (Patzelt *et al.,* 2001).

2.2.4 PrsA protein

PrsA protein (protein secretion) was discovered in the isolation of mutants defective in protein export (Kontinen and Sarvas 1988). A *B. subtilis* strain secreting high amount of α-amylase of *B. amyloliquefaciens* (AmyQ) was mutagenized with NNG (N-methyl-N-nitroso-N-nitroguanidine) and screened for mutants with decreased secretion of AmyQ on starch plates. Mutations were mapped into four loci by transduction with PBS1 bacteriophage and transformation. One mutation, which mapped very close to the *glyB133* marker, was named *prs-3*. This mutation decreased the secretion of AmyQ dramatically; at the stationary phase of growth only 2% of AmyQ was secreted into the culture medium compared to wt. The *prs-3* mutation also decreased the amount of secreted exoprotease but had no affect on the secretion of lipopenicillinase.

A gene locus identified by the *prs-3* mutation was cloned from the genomic lambda library and designated *prsA* (Kontinen *et al.,* 1991). The *prsA* gene is a monocistronic open reading frame of 876 nucleotides encoding a protein of 292 amino acid residues. PrsA is an exported protein and has a 19 aa long N-terminal signal peptide with a lipoprotein signal peptidase cleavage-site. The precursor size of PrsA is 32.5 kDA resulting in a 30.5 kDa mature protein. The *prs-3* mutation is a missense mutation replacing Asp_{249} with Asn_{249} (numbering according the mature

form). This mutation results in a degradation-prone variant but does not influence PrsA activity *in vivo*; the phenotype of *prsA3* is due to a low level of PrsA3 protein (Hyyryläinen *et al.,* 2000). PrsA is lysine rich (18.7%) giving the protein a hydrophilic nature, a high pI (8.5) and a positive net charge of +3. Yet, there is only one cluster of lysines in the protein, most of the lysines spreading singly throughout the entire sequence. Another special feature of PrsA is the serine/threonine rich region of 15 aa (serine tail) at the very end of the C-terminus. Characteristically, in *B. subtilis* polyserine sequences are found in proteins that are predicted to have interactions with the cell wall. According to secondary structure predictions, most of PrsA is α-helix with some β-sheet formation in the middle part of the protein. There are no membrane-spanning motifs in PrsA (Kontinen *et al.,* 1991 and the TMPRED program in TMbase, http://www.ch.embnet.org).

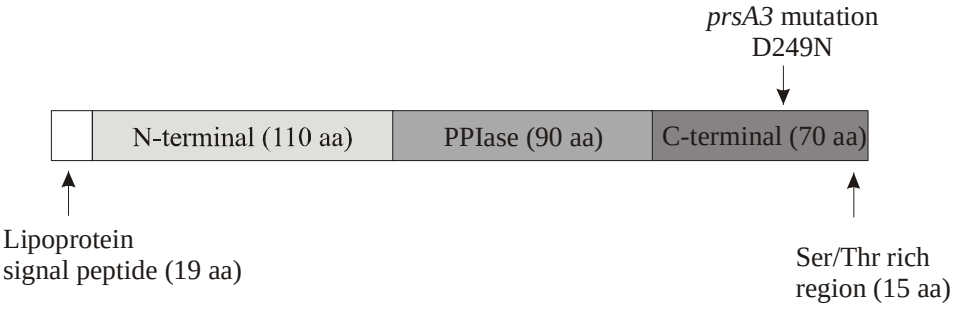


Figure 3. Schematic representation of the PrsA lipoprotein. Three domains are marked as N-terminal, PPIase and C-terminal. The signal peptide and serine/threonine rich region are also shown. The position of the prsA3 mutation is marked with an arrow.

PrsA seems to be a three-domain protein (Figure 3). The middle region of PrsA, the PPIase domain (~ 90 aa), shares high sequence similarity with the parvulin-type PPIases (Rudd *et al.,* 1995). The identity of PrsA with *E. coli* Parvulin, Pin1 and hPar14 is 40, 52 and 41%, respectively. However, the flanking N-terminal (~ 110 aa) and C-terminal domains (~ 70 aa) do not share similarities with any characterized proteins. The N-terminal domain shares sequence similarities only with PrsA proteins of closely related Gram-positive bacteria, and in the case of the C-terminal domain there are hardly any similarities even between the C-terminal domains of PrsA proteins from different species (Sarvas *et al.,* 2004).

PrsA homologs have been identified in other *Bacillus* species as well as in Gram-positive bacteria such as *Lactococcus, Listeria, Lactobacillus, Staphylococcus,*

Streptococcus and *Clostridium* (Vos *et al.*, 1989; Glaser *et al.*, 2001; Kuroda *et al.*, 2001; Nolling *et al.*, 2001; Terada *et al.*, 2001; Tettelin *et al.*, 2001; Kleerebezem *et al.*, 2003). All PrsAs are modular proteins with the middle part sharing sequence similarity with parvulin-type PPIases (Sarvas *et al.*, 2004). Some of these proteins have been characterized. *B. anthracis* has three *prsA* homologs (Tettelin *et al.*, 2001; Read *et al.*, 2003). When expressed in *B. subtilis*, all these proteins complemented the *B. subtilis prsA* (Williams *et al.*, 2003). *B. cereus* strain UW85 has a PrsA homolog LipA whose expression is induced when cells are exposed to plant exudates suggesting that LipA plays a role in interaction with the host plant (Dunn *et al.*, 2003). Two PrsA homologs have been identified in lactococci: PrtM is required for the processing of a PtrP protease (Vos *et al.*, 1989; Haandrikman *et al.*, 1991) and PmpA stabilizes the secretion of some proteins (Drouault *et al.*, 2002).

2.2.4.1 PrsA is a membrane-bound lipoprotein

Subcellular fractioning of cells overexpressing PrsA revealed that essentially all PrsA was found in the membrane fraction (Kontinen and Sarvas 1993). Since there are no predicted membrane spanning motifs in PrsA, the membrane association needs to occur with an optional manner. N-terminal lipidation of proteins is a major mechanism in tethering proteins to the membrane in bacteria (Sutcliffe and Russell 1995). This is specifically important in Gram-positive bacteria since the lack of the outer membrane and the porous structure of the peptidoglycan require attachment of proteins to the membrane to prevent their loss into the culture medium. Lipidation of proteins is directed by the lipobox sequence (Leu_{-3}-Ala/Ser_{-2}-Gly/Ala_{-1}-Cys_{+1}) at the 3′ end of the signal sequence (Sutcliffe and Harrington 2002). This lipobox consensus sequence is found in the PrsA signal peptide as Leu_{-3}-Ser_{-2}-Ala_{-1}-Cys_{+1} (Kontinen *et al.*, 1991). Followed by the translocation of a prelipoprotein, an enzyme called prolipoprotein diacylglycerol transferase (Lgt) catalyses the addition of diacylglycerol into the thiol group of cysteine within the lipobox. Phospholipids are used as substrates in the reaction. Subsequently, the signal peptide is cleaved off by the lipoprotein signal peptidase Lsp. After the removal of the signal peptide, the cysteine with the lipid anchor is the first residue of the mature protein. Cell labeling studies have confirmed the lipoprotein nature of PrsA (Kontinen and Sarvas 1993). These labeling studies have also shown the necessity of the *lgt* gene for lipidation of PrsA. In an *lgt* mutant PrsA remained in the nonlipomodified form and its release into the culture medium increased (Leskelä *et al.*, 1999a).

2.2.4.2 Function of PrsA

The mode of action of PrsA is still unknown. Data indicate that PrsA is involved in the late stages of secretion, namely in the posttranslocational folding of secretory proteins but the detailed molecular mechanism of how PrsA facilitate the folding is

unclear. The PrsA3 mutant has been a valuable tool for studying the function of PrsA. Due to the amino acid substitution PrsA3 is susceptible to proteolysis reducing its amount more than 10-fold. The *prsA-3* mutation decreased the secretion of AmyQ α-amylase (2% of the wt level) expressed at a high level whereas the effect was less profound (51% of the wt level) when α-amylase was chromosomally encoded and therefore expressed at a lower level (Kontinen and Sarvas 1988). The secretion of α-amylase expressed at a high level is known to be limited by the capacity of the cellular secretion machinery (Sibakov 1986). To elucidate whether PrsA is one of the limiting factors, the effect of PrsA overexpression on three exoproteins expressed in *B. subtilis* was tested (Kontinen and Sarvas 1993). PrsA overexpression increased the secretion of two α-amylases, AmyQ of *B. amyloliquefaciens* and AmyL of *B. licheniformis*, 2.5- and 6-fold, respectively. PrsA also increased the secretion of subtilisin protease of *B. licheniformis* by 2-fold. These data suggested that PrsA is a bottleneck in secretion and could be a factor to modify and increase the capacity of the secretion machinery. Moreover, attempts to inactivate *prsA* gene failed indicating the essential function of the protein (Kontinen and Sarvas 1993).

Since subtilisin is a PrsA dependent protein, subtilisin-alkaline phosphatase (SubC'-'PhoA) fusions were used as protein models in kinetic studies by pulse-chase experiments (Jacobs *et al.*, 1993). Alkaline phosphatase (AP) activity in culture medium was measured to detect the activity of the fusion protein. In the *prsA3* mutant expressing a SubC(pre)-PhoA fusion the AP activity was decreased by half. Consistent with the enzymatic activity, the PhoA amount released into medium in pulse chase experiments was also reduced. Fusion proteins containing the prosequence of SubC caused even more dramatic effects; in the *prsA3* mutant most of the synthesized fusion protein was degraded during the pulse-chase and only a small fraction was chased into the mature form. Cell fractioning experiments revealed that the degradation was dominantly cell-associated. These data indicated misfolding of the model protein in *prsA3* cells and the involvement of PrsA in the posttranslocational folding (Jacobs *et al.*, 1993).

The cell wall microenvironment affects the requirement of PrsA for protein folding (Hyyryläinen *et al.*, 2000; Wahlström *et al.*, 2003). Secretion and stability of the model protein AmyQ was compared wt and *prsA3* cells and in protoplasts, respectively. In cells AmyQ secretion is strictly PrsA-dependent and therefore less AmyQ was secreted in *prsA3* cells than in wt cells. However, in protoplasts devoid of the cell wall the same amount of AmyQ was secreted in wt and *prsA3* mutant. Furthermore, the trypsin sensitivity of AmyQ secreted from the *prsA3* protoplasts was similar to AmyQ secreted from wt protoplasts suggesting that AmyQ is correctly folded in both cases. These results indicate that the posttranslocational

folding of AmyQ is PrsA-independent in the absence of the cell wall (Wahlström *et al.*, 2003). The net charge of the cell wall has an effect on PrsA3 as well. A *dlt* mutation (see section 2.3.2 below) that results in an increase in the negative charge of cell wall polymers rescued PrsA3 from degradation and resulted in an increased amount of PrsA3, which in turn enhanced the secretion of PrsA-dependent proteins. The *dlt* mutation also partially suppressed the lethal effect of *prsA* depletion (Hyyryläinen *et al.*, 2000). The mechanism by which increased negative cell wall charge stabilizes PrsA3 is unknown; possibly the rate of folding of PrsA3 could be improved, or unfolded PrsA3 as a positively charged protein could bind better to more negative polymers of the cell wall and for that reason be rescued from degradation or, maybe the modified cell wall might inhibit the protease responsible for degradation (Hyyryläinen *et al.*, 2000).

2.3 Cell wall

The major difference between Gram-negative and positive bacteria is the number of cell membranes and the cell wall structure. While Gram-negative bacteria possess an inner and outer membrane and the periplasmic space located between surrounded by the cell wall of 1 to 2 peptidoglycan layers Gram-positive bacteria have only a single cytoplasmic membrane surrounded by 10 to 20 layers of peptidoglycan containing covalently linked anionic polymers (Foster and Popham 2002) and below). This thick cell wall of 30-40 nm is the final barrier for the release of exported proteins into the medium. A polyanionic matrix with ion-exchange properties provides functions relating to cell shape, maintaining cation homeostasis and trafficking of cations, nutrients, proteins and antibiotics. In Gram-positive bacteria, the cell wall-membrane interface can by considered functionally similar to the periplasmic space of Gram-negative bacteria since it is the cell compartment in which several cellular processes, corresponding to those found in the periplasm of Gram-negative bacteria, take place.

2.3.1 Composition of the cell wall

Peptidoglycan structure of the cell wall consists of long glycan strands of disaccharide residues of N-acetyl glucosamine and N-acetylmuramic acid. The chain length varies; in *B. subtilis* it is approximately 100 disaccharides (Ward 1973). Short peptides are linked to the carboxyl of the muramic acid residues cross-linking the disaccharides (Foster and Popham 2002). Peptidoglycan precursor synthesis occurs

in the cytoplasm in a stepwise-reaction facilitated by a great number of enzymes resulting in a lipid-linked disaccharide-pentapeptide precursor called lipid II. The genes involved in the peptidoglycan synthesis are best-characterized in *E. coli*. Homologous genes have been assigned in *B. subtilis* and demonstrated to be essential (Foster and Popham 2002). Lipid II precursors are polymerised into glycan strands by glycosyl transferases, cross-linked by transpeptidases and cleaved by carboxypeptidases; these enzymatic activities are carried out by penicillin-binding proteins (PBPs) in the cell wall (Ghuysen 1991). New peptidoglycan layers are inserted into the cell wall by moving old layers from the inside to the outside and finally shedding them into the culture medium and degrading them (Foster and Popham 2002).

There are two main categories of anionic polymers attached to the cell wall, teichoic and teichuroic acids (Neuhaus and Baddiley 2003). Altogether, anionic polymers make up 35-60% of the dry weight of the cell wall in *B. subtilis*. In *B. subtilis* 168, the major form of wall teichoic acids (WTA) is glycerol phosphate (Gro-P) polymer (length 45-60), in which a hydroxyl group is often substituted by a glucose molecule or a D-alanine residue (Baddiley 1970; Neuhaus and Baddiley 2003). Under phosphate-limiting conditions, non-phosphorus teichuronic acids are synthesized instead of teichoic acids. Altogether, WTA plays an essential role in the growth and morphology of *B. subtilis.* Precursors for the WTA biosynthesis are produced as part of the general metabolism and the assembly of WTA occurs mainly within the membrane by enzymes encoded by the *tag* genes (Mauel *et al.*, 1994; Soldo *et al.*, 1999). Lipoteichoic acids (LTA) of *B. subtilis* are polymers of glycerol phosphate attached to the glycolipid anchor and are mainly associated with the cytoplasmic membrane instead of the cell wall. The biosynthesis of LTA involves lipid carriers but the mechanism is poorly understood and not genetically elucidated (Foster and Popham 2002).

2.3.2 Teichoic acids and D-alanylation

One of the main determinants of the net anionic charge of the cell wall in Gram-positive bacteria are the D-alanyl esters (D-Ala) covalently linked to teichoic acids (Neuhaus and Baddiley 2003). D-alanyl esterification decreases the negative charge of the cell wall and concomitantly the capacity to bind cations decreases (Lambert *et al.*, 1975a; 1975b). The content of D-Ala in LTA and WTA is highly variable; the molar ratio of D-Ala to phosphate in LTA varies from species to species from being not detectable to 0.88 (Neuhaus and Baddiley 2003). Generally, WTA has a lower ratio than LTA. The degree of D-Ala content is a function of several conditions. An

alkaline pH in the growth medium reduces the ester content dramatically since D-alanyl esters are highly labile in elevated pH (pH > 7.5) (Ellwood and Tempest 1972; MacArthur and Archibald 1984). In addition to pH, elevated temperature and increasing concentration of NaCl decrease the D-alanylation (Novitsky *et al.*, 1974; Fischer *et al.*, 1981).

The D-alanylation of LTA has been well elucidated (Figure 4). The synthesis of D-alanyl-LTA requires four proteins encoded by the *dlt* operon characterized in several species (Neuhaus and Baddiley 2003). D-alanyl carrier protein ligase (Dcl) and D-alanyl acyl carrier protein (Dcp) are encoded by *dltA* and *dltC*, respectively. In the two-step ATP-consuming reaction Dcl forms a D-alanyl AMP intermediate and transfers the alanyl residue from AMP to the carrier protein Dcp (Perego *et al.*, 1995) that donates the alanyl residue to the poly Gro-P moiety of LTA (Heaton and Neuhaus 1994). DltB is a putative transport protein and is assumed to function in the export of D-alanyl-Dcp across the cytoplasmic membrane (Neuhaus and Baddiley 2003). The membrane protein DltD functions in the selection of the correct carrier protein for the ligation and in the hydrolysis of mischarged D-alanyl-ACPs (Debabov *et al.*, 1996; Debabov *et al.*, 2000). The *dlt* operon of *B. subtilis* has an additional fifth gene, *dltE*, which encodes an oxidoreductase that is not required for the D-alanylation (Perego *et al.*, 1995). The *dlt* operon in *B. subtilis* is part of the σ^x regulon that regulates a variety of genes affecting the wall cell composition and metabolism (Huang *et al.*, 1997; 1998). Additionally, the *dlt* operon is under the control of the SpoOA and AbrB regulatory systems involved in regulation of sporulation (Perego *et al.*, 1995).

The physiological function of D-alanylation is quite unclear. It has been proposed to modulate the activities of autolysins, to maintain cation homeostasis and to define the electromechanical properties of the cell wall (Neuhaus and Baddiley 2003). Loss of D-alanylation increases the binding capacity of the cell wall to cations e.g. Ca^{2+} and Mg^{2+} and to basic proteins as well (Lambert *et al.*, 1975a), therefore D-alanine might be a component of a regulatory system. D-alanylation deficient mutants have been identified in several species and D-alanylation appears not to be essential for viability. In *B. subtilis*, inactivation of *dltA*, *dltB*, *dltC* or *dltD* resulted in the loss of D-alanylation in both LTA and WTA (Perego *et al.*, 1995). Other phenotypic effects were minor, only enhanced autolysis and increased susceptibility to methicillin was detected (Wecke *et al.*, 1996; 1997). However, D-alanylation deficiency had an impact on the stability of several proteins in *B. subtilis*. Inactivation of *dltD* suppressed *prsA3* mutation and restored the secretion deficiency caused by mutant PrsA3 protein (Hyyryläinen *et al.*, 2000 and section 2.2.4.2). In a *dlt* mutant, heterologous proteins or abnormal secreted proteins prone to degradation after translocation were stabilized and secreted in increased quantities (Hyyryläinen *et al.*,

2000; Thwaite *et al.*, 2002; Craynest *et al.*, 2003). Cations are known to increase the folding of some secreted proteins including α-amylases and levansucrases (Petit-Glatron *et al.*, 1993; Stephenson *et al.*, 1998), and thus the increased binding of cations in the cell wall in a *dlt* mutant might be the explanation for the enhanced stability of proteins. Interestingly, the *dlt* mutation was also able to partially suppress the lethal effect of the *prsA* depletion (Hyyryläinen *et al.*, 2000). In other Gram-positive species besides *Bacillus*, the *dlt* mutation has a more dramatic effect. In *Staphylococcus* species, the inactivation of *dlt* increased the sensitivity of bacteria to antimicrobial cationic peptides (Peschel *et al.*, 1999), and in *Streptococcus* species lower growth rate, acid sensitivity, defective cell separation and synthesis of intracellular polysaccharides were detected (Spatafora *et al.*, 1999; Boyd *et al.*, 2000).

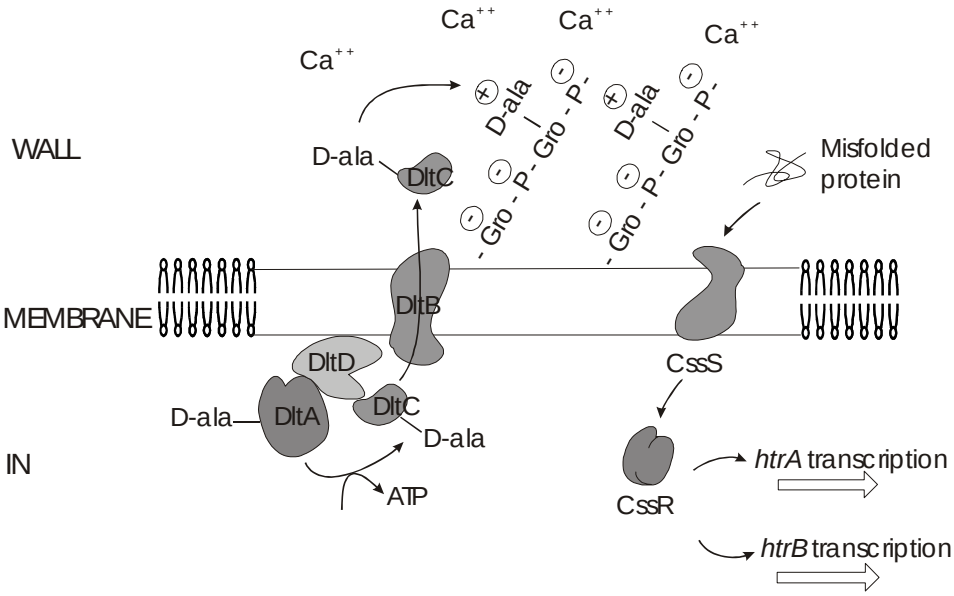


Figure 4. Model for incorporation of the D-alanyl into membrane associated LTA (left side) and model of the CssRS two-component regulatory system in B. subtilis (right side). Incorporation of D-alanyl into LTA: DltD provides binding sites for D-alanyl acyl carrier ligase (DltA) and D-alanyl carrier protein (DltC). DltB provides a putative channel for the transport of DltC across the cytoplasmic membrane to the outer surface of the membrane in which DltC donates the D-alanyl to the Gro-P moiety of LTA.

CssRS two-component system: Sensor kinase CssS in the membrane senses the accumulation of misfolded proteins caused by secretion stress. In response to the secretion stress CssS autophoshorylates and then transfers the phosphate to the cytoplasmic response regulator CssR, which in turn activates the transcription of genes encoding HtrA-type proteases. HtrA-type proteases as quality control proteases then degrade the misfolded protein in the membrane-cell wall interface.

2.3.3 Cell wall-bound proteins

In *B. subtilis* the cell wall contains approximately 10 % of the total protein content (Pooley *et al.,* 1996). These cell wall-bound proteins (CWBPs) include nucleases, proteases, penicillin binding proteins and wall cell hydrolases involved in various functions in the cell wall (Merchante *et al.,* 1995; Margot and Karamata 1996; Blackman *et al.,* 1998; Antelmann *et al.,* 2002; Popham and Young 2003; Scheffers *et al.,* 2004). Many CWBPs of Gram-positive bacteria are anchored to the cell wall by a transpeptidation mechanism requiring a C-terminal sorting signal LPXTG and an enzyme called sortase (Navarre and Schneewind 1999). However, *Bacillus* CWBPs do not contain LPXTG sorting signals but proteins are attached to the cell wall by electrostatic forces (Foster and Popham 2002). In addition to the N-terminal signal, many CWBPs are characterized by specific cell wall retention signals, a variable number of non-catalytic domains often containing tandem repeats, which have an affinity for the components of the cell wall (Ghuysen *et al.,* 1994; Baba and Schneewind 1998). Potential cell wall-binding motifs are also present in some predicted membrane proteins e.g. in HtrA protease and YclI transporter suggesting that these proteins are active in contacts between the membrane and the cell wall (Tjalsma *et al.,* 2000). It is still unclear which cell wall components are involved in targeting the CWBPs into the cell wall in *B. subtilis.*

The best-characterized CWBPs in *B. subtilis* are penicillin-binding proteins (PBPs) that mediate the polymerisation and cross-linking of peptidoglycan (see above). PBPs were discovered and named for their affinity for penicillin that mimics the pentapeptide procursor side chain structure and blocks wall synthesis (Suginaka *et al.,* 1972; Spratt and Pardee 1975). The PBPs can be classified into three groups (Ghuysen 1991; Goffin and Ghuysen 1998): class A high-molecular-weight (high MW) bifunctional PBPs with both a transglycosylase and a transpeptidase domain, class B high-MW PBPs with an N-terminal domain of unknown function and a transpeptidase domain and low-molecular-weight (low-MW) PBPs with carboxy- or endopeptidase activity. PBPs function in both vegetative growth and in the formation of endospores with redundancy in their function (McPherson *et al.,* 2001; McPherson and Popham 2003). There are 16 PBPs known in *B. subtilis* and most

of them have been identified biochemically as well (Foster and Popham 2002). There is only one known essential PBP of *B. subtilis*, class B PBP2b, which plays a specific role in the cell division (Daniel *et al.*, 2000).

Autolysins are hydrolysing enzymes that digest the cell wall peptidoglycan. Selective degradation of peptidoglycan is required for various functions, e.g. for cell wall turn-over, cell division, sporulation and germination (Smith *et al.*, 2000). Autolysins can be classified as muramidases, glucosaminidases, amidases and endopeptidases according to their hydrolytic bond specificity (Ghuysen *et al.*, 1966). In *B. subtilis* 35 autolysin genes have been identified and clustered into 11 families (Kunst *et al.*, 1997). Similar to PBPs the functional redundancy is evident. The major autolysins of vegetative growth, the amidase LytC and glucosaminidase LytD, have been most studied at the molecular level (Smith *et al.*, 2000). Together LytC and LytD account for about 95% of the autolytic activity of the cell (Blackman *et al.*, 1998).

The cell wall also contains proteases and is thus a site for proteolytic degradation. In *B. subtilis*, several extracellular proteases of two major categories, serine proteases and metalloproteases, have been identified (Kunst *et al.*, 1997). Most of these proteins are secreted into the medium but some e.g. serine protease WprA, remain cell wall-associated and are eventually released into the medium as a consequence of wall turn-over (Stephenson and Harwood 1998). The primary products of *wprA* is CWBP52 serine protease and CWBP23 protein assumed to regulate the activity of CWBP52 (Margot and Karamata 1996; Babe and Schmidt 1998; Stephenson and Harwood 1998; Bolhuis *et al.*, 1999b; Corvey *et al.*, 2003). CWBP52 has been shown to be involved in the degradation of a heterologous α-amylase and the mutant SipS, and in the processing of peptide antibiotic subtilin (Stephenson and Harwood 1998; Bolhuis *et al.*, 1999b; Corvey *et al.*, 2003).

2.3.3.1 HtrA-type proteases

Membrane- and cell wall-associated proteins are a part of the quality control system of the exported proteins. Under environmental or secretion stress conditions the accumulation of misfolded proteins in the periplasm or at the membrane-cell wall interface induces the expression of the cleaning proteases that degrade the misfolded and aberrant proteins. One of the cleaning proteases is the HtrA family of serine proteases found widely in both Prokarya and Eukarya (Clausen *et al.*, 2002). HtrA proteases are composed of a catalytic core domain and one or more C-terminal PDZ-domains required for the assembly of protein monomers into a functional hexameric complex (Sassoon *et al.*, 1999; Clausen *et al.*, 2002). The HtrA hexamer is formed

by staggered trimeric rings, in which the proteolytic sites are located in the inner cavity, and the PDZ-domains in the complex mediate the substrate binding and opening and closing of the cavity (Krojer *et al.*, 2002).

There are three HtrA-like proteases in *E. coli*, HtrA (DegP), HhoA (DegQ) and HhoB (DegS) (Strauch and Beckwith 1988; Lipinska *et al.*, 1989; Bass *et al.*, 1996; Waller and Sauer 1996). DegP is a periplasmic protease that degrades misfolded proteins generated under various stress conditions. Additionally, DegP has been shown to participate in bacterial virulence (Pallen and Wren 1997). Yet, DegP is not solely a protease but it also possesses a general chaperone activity (Spiess *et al.*, 1999). There is a temperature-dependent switch of these activities: in low temperature a chaperone activity dominates while the proteolytic activity is present in elevated temperatures. The *degP* gene is regulated by two signal transduction pathways, the Cpx- and the σ^E-pathway (Danese and Silhavy 1997; Pogliano *et al.*, 1997). The Cpx-pathway is a two-component system being composed of an inner membrane sensor kinase CpxA and a response regulator CpxR. In the phosphorylated form CpxR stimulates transcription of possibly hundreds of genes including thiol-disulphide oxidoreductases, PPIases and proteases (De Wulf *et al.*, 2002). Phosphorylation of CpxR is controlled by CpxA, which acts both as a kinase and phosphatase and is in turn regulated by CpxP. Lipoprotein NlpE also upregulates the Cpx-system (DiGiuseppe and Silhavy 2003).

In *B. subtilis* there are three HtrA-type proteases, HtrA (YkdA), HtrB (YvtA) and YyxA (Kunst *et al.*, 1997; Tjalsma *et al.*, 2000). All three proteins are predicted to span the membrane with the N-terminus located in the cytoplasm and the catalytic domain and the single C-terminal PDZ-domain located membrane-cell wall interface. Two of proteases, HtrA and HtrB, display many similarities; their expression is low at the exponential phase of growth, increases in the stationary phase of growth and both are induced by heat or secretion of heterologous α-amylase suggesting overlapping cellular functions of these two proteases (Noone *et al.*, 2001). In response to secretion stress HtrA is present at an elevated level in the extracellular proteome (Antelmann *et al.*, 2003). In contrast to HtrA and HtrB, the expression of YyxA is low at the exponential phase of growth, it decreases at the stationary phase of growth and is not induced by heat or secretion stress (Noone *et al.*, 2001). Both HtrA and HtrB are regulated by the CssRS (control of secretion stress regulator and sensor) two-component system, which is the *B. subtilis* homolog of the *E. coli* CpX-system and determines the level of proteolytic activity in the cell envelope (Figure 4) (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). CssS is the membrane sensor kinase and CssR the response regulator component. The CssRS system is required in secretion stress conditions, such as in the condition of reduced level of PrsA (Hyyryläinen *et al.*, 2001). Altogether, the CssRS-regulated genes *htrA*

and *htrB* represent class V of heat-inducible genes in *B. subtilis* (Darmon *et al.*, 2002).

2.4 Genetic engineering of components involved in the late stages of secretion in *B. subtilis*

B. subtilis has several advantages as a production host for heterologous proteins. Since it is a Gram-positive bacterium, proteins need to be translocated across a single membrane into the extracellular environment. Additionally, *Bacillus* has a high capacity to secrete its own exoproteins indicating that it has efficient natural secretion machinery. Secretion permits accumulation of products at a high level into the medium and thus simplifying product recovery. Fermentation and technology for large-scale cultivations have been well developed over the years. Morever, *B. subtilis* and some other *Bacillus* species lack pathogenicity and endotoxins and are regarded as GRAS organisms (Generally Regarded As Safe). To improve the secretion of heterologous proteins in *B. subtilis*, there has been intensive research on the secretion machinery over the years. Some heterologous proteins can indeed be secreted at high level in *Bacillus*, such as α-amylase of *B. amyloliquefaciens* (1-3 g/l) (Palva 1982), protein A of *S. aureus* (1 g/l) (Fahnestock and Fisher 1987) and human interleukin IL-3 (0,1 g/l) (van Leen *et al.*, 1991) but in most cases the yields have been disappointingly low. Low yields in protein secretion indicate that there are bottlenecks in the secretion pathway. Problems for efficient protein production have been identified in all steps, starting from plasmid instability and ending in the posttranslocational events.

Correct folding and avoidance of proteolytic degradation are the key elements in posttranslocational events. To overcome the degradation problem, strains with low protease activity have been constructed by deleting known extracellular protease genes. In many cases, better protein yields have been obtained in these protease deficient strains (Fahnestock and Fisher 1987; Wu *et al.*, 1991; Ye *et al.*, 1999; Wu *et al.*, 2002). In addition to secreted proteases, the proteases residing in the membrane-cell wall interface are problematic (Meens *et al.*, 1997; Stephenson and Harwood 1998; Jensen *et al.*, 2000). Consequently, the disruption or lowered expression of the *wprA* gene encoding the cell wall-bound serine protease increased stability and yield of several heterologous secretory proteins, e.g. α-amylase, staphylokinase, recombinant lipase and single chain antibody (Stephenson and Harwood 1998; Kobayashi *et al.*, 2000; Lee *et al.*, 2000; Wu *et al.*, 2002). The components of the cell wall may be problematic as well. Thus inactivation of *dlt* genes resulting in the absence of D-alanylation of the cell wall is a beneficial factor

for secretion of some heterologous proteins (Hyyryläinen *et al.*, 2000; Thwaite *et al.*, 2002; Craynest *et al.*, 2003). In a *dlt* mutation the yields of α-amylase, glycosyltransferase and secreted antrax protective antigen increased 2-, 4- and 2.5-fold, respectively. Though the separate inactivation of *wprA* and *dlt* was beneficial, the simultaneous inactivation of the genes reduced the yield of α-amylase (Stephenson *et al.*, 2002).

When heterologous proteins are expressed at high levels, the amount of proteins assisting folding becomes inadequate for correct folding. Therefore, overexpression of these folding factors is a means to improve the yield. Several studies show that heterologous proteins are secreted at enhanced levels when components involved in posttranslocational folding are modulated (see below). In addition to this, an increased amount of cytosolic chaperones may prevent the aggregation of secretory proteins in the cytoplasm prior to translocation. Secretion of two single-chain antibodies (scFv) was increased in *B. subtilis* through overproduction of GroEL-GroES and DnaK-DnaJ-GrpE chaperone machineries (Wu *et al.*, 1998; Wu *et al.*, 2002). Thiol-disulphide oxidoreductases and PPIases are limiting factors for folding. So far, there is no data on the effect of thiol-disulphide oxidoreductases on secretion of heterologous proteins in *B. subtilis* but in *E. coli* increased amount of Dsb proteins enhanced stability and production of several proteins containing disulphide bonds (Kurokawa *et al.*, 2000; 2001; Zhang and Huang 2002). The soluble portion of scFv increased 50% to 90% when DsbC or DsbD was fused with scFv and overprodused simultaneously. Moreover, the overexpression of *dsbABCD* increased the periplasmic production of horseradish peroxidase and human nerve growth factor 7- and 3-fold, respectively.

PrsA protein, the only extracytoplasmic PPIase homolog in *B. subtilis*, has no doubt potential of being a protein to enhance protein secretion. Already the first studies on PrsA identified it as a bottleneck for secretion of some exoproteins of *Bacillus* species (see section 2.2.4.2). Protective antigen of *B. anthracis* is also a protein dependent on PrsA; its production increases as a function of PrsA concentration (Williams et al., 2003). PrsA overproduction does not only improve the secretion of bacillar proteins, it also increases the secretion of a eukaryotic protein, antidigoxin single chain antibody (SCA) (Wu *et al.*, 1998). Secretion of SCA increased 1.6-fold, and simultaneous overexpression of *prsA* and cytosolic chaperones further improved the yield resulting in a 3-fold increase in secretion up to 12 mg/l. The data clearly demonstrate a role for PrsA in biotechnical applications.

3 AIMS OF THE STUDY

PrsA lipoprotein is an essential component in the posttranslocational folding of secreted proteins of *B. subtilis*. PrsA is crucial for efficient secretion of several exoproteins and therefore has special importance and potential in biotechnology for the production of valuable native and heterologous proteins. In this study, the aim was to further characterize the PrsA protein and its role in the posttranslocational folding and secretion in *B. subtilis*. In addition to the PrsA protein, two other factors affecting the late stages of protein folding were studied, namely the negative net charge of the cell wall and the HtrA-type quality control proteases in the membrane-cell wall matrix.

Specific aims of this study were:

- To demonstrate that *prsA* is essential for viability.
- To show the PPIase activity of PrsA *in vitro* and by mutagenesis studies relate this enzymatic activity to the *in vivo* function.
- To study the effect of PrsA depletion on the exoproteome.
- To study the saturation of secretion machinery and the role of PrsA as a limiting factor.
- To study the effect of three factors affecting posttranslocational protein folding and degradation on secretion of heterologous proteins. These factors were PrsA level, increased negative net charge of the cell wall and depletion of HtrA-type quality control proteases.

4 MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in the articles.

Table 2. Methods used in this study

Method	Described and used in
α-amylase assay of culture medium	I, II, III
α-amylase halo assay on culture plates	I
β-glucanase assay	III
β-lactamase assay	III
Complementation of PrsA depletion	I
Determination of specific secretion rate and number of protein molecules/cell	II
Gram staining and microscopy	II
Immunoblotting	I, II, III
Molecular cloning techniques	I, II, III
Molecular modeling	I
Protease accessibility assay	I, II
Protease-coupled PPIase assay	I
Purification of GST-PrsA fusion proteins	I
Purification of lipo-PrsA	I
Purification of non-lipomodified PrsA	I
Rhodanese refolding assay	I
Ribonuclease T1 refolding assay	I
SDS-PAGE	I, II, III
Site-directed mutagenesis	I
Strain construction techniques	I, II, III
Transposon mutagenesis	I

5 RESULTS AND DISCUSSION

5.1 PrsA is essential for viability (II)

Previous attempts to disrupt the *prsA* gene were unsuccessful suggesting that the gene was essential (Kontinen *et al.*, 1991). This hypothesis was studied more thoroughly by placing the chromosomal *prsA* under IPTG (isopropyl-β-D-thiogalactopyranoside) inducible P_{spac} promotor, which resulted in a tightly controlled expression of *prsA*. A fragment of *prsA* was cloned into pMUTIN4 plasmid (Vagner *et al.*, 1998) and integrated into the chromosome by a single crossing over in the presence of IPTG. The integration resulted in the expression of *prsA* from the P_{spac} promotor of pMUTIN4. The viability of the constructed strain (IH7211) was then studied by calculating the viable count of cells and examining cell morphology. Bacteria from one colony were suspended into water, diluted and plated out onto plates with and without IPTG. Colonies growing on IPTG-containing plates had morphologies identical to the wt and the viable count 3×10^7/ml was comparable to wt. However, in the absence of IPTG the colonies were heterogenous in size most appearing very small and the viable count was only 8×10^3/ml (0.01 % of the wt). These results clearly indicate that *prsA* is essential for cell viability.

The appearance of colonies on plates without IPTG indicates the presence of a suppressor mutation that allows some growth even in the absence of PrsA. Furthermore, the heterogenous morphology of these colonies suggests the presence of several different suppressor mutations. A known suppressor of *prsA* is a mutation in the *dlt* operon responsible for D-alanylation of the teichoic acid in the cell wall (Hyyryläinen *et al.*, 2000 and section 2.3.2). Since the requirement of PrsA is connected to the cell wall (Wahlström *et al.*, 2003 and section 2.2.4.2) mutations in other genes affecting the cell wall synthesis and composition beside the *dlt* operon are potential suppressor candidates. Additionally, mutations in genes encoding membrane or cell wall-bound proteases might also be hypothetical suppressors. Decreased proteolysis due to a mutated protease would allow more time for folding without degradation and therefore folding could perhaps take place in the absence of PrsA. However, in this study the colonies harbouring potential suppressors mutations were not further studied.

Next, we determined the lowest level of PrsA protein able to maintain normal growth. IH7211 cells were cultivated at different IPTG concentrations, the cells were Gram-stained and morphology was observed by microscopy. At 24 μM IPTG induction the growth was still quite normal. However, 16 μM IPTG or lower

induction was not enough for normal growth since morphological changes in cells were detected. Whereas wt cells grow as short chains of few cells, cells grown at 16 μM IPTG formed long filaments. At 8 μM IPTG level the abnormalities became even more serious; cells were enlarged and spherical, and appeared to be fragile as indicated by the presence of cell debris. At 1-2 μM IPTG no growth was observed. These morphological changes in the cells depleted of PrsA resemble the phenotype observed when the synthesis of cell wall polymers is deficient. There is both filamentous growth and cell lysis in mutants deficient in peptidoglycan synthesis (PBP mutants) (Popham and Setlow 1996; Murray *et al.*, 1998; Foster and Popham 2002). Spherical cells also appear if synthesis of wall teichoic acids is deficient (Lazarevic and Karamata 1995). The similarities in phenotype suggest that PrsA has a role in the folding of enzymes involved in the synthetic pathway of the cell wall. Since PrsA is an essential protein for viability it indicates that PrsA is needed for the folding of essential proteins in the synthesis of peptidoglycan and/or teichoic acids.

We calculated the number of PrsA molecules in a wt cell and the PrsA amount needed to maintain normal growth. By using purified PrsA protein as a standard in quantitative western blotting and determining the cell count of the sample, we were able to estimate the number of PrsA molecules per cell. PrsA turned out to be a very abundant protein; wt cells contain about 20 000 PrsA molecules. Induction at 24 μM IPTG was used to calculate the PrsA number that was enough to support normal growth. Only 1 % (about 200 PrsA molecules per cell) was able to support normal growth although the cells did form filaments indicating the minimal threshold for normal growth. The number of PrsA molecules clearly outnumbers those of Sec translocase complexes. In *E. coli*, estimates of the number of Sec translocases on the membrane varies from 30 to 900 molecules per cell (Matsuyama *et al.*, 1990; Mizushima 1992; Pogliano *et al.*, 1997). A similar quantitation has not been carried out in *Bacillus* but if the cellular levels are in the same range as in *E. coli*, there is a high excess of PrsA compared to the translocase. Therefore, it seems unlikely that PrsA would be in a stoichiometric way associated with the translocase complex. The large number of PrsA molecules and free lateral fusion of the membrane possibly ensures that there is enough PrsA to interact with translocated proteins even without any specific association with the translocase complex.

5.2 All domains of PrsA are essential for its function *in vivo* (I)

To identify which regions of PrsA are required for secretion and viability, a set of *prsA* deletion mutations was constructed by PCR and the mutations were characterized for their effects on the *in vivo* PrsA activity by two complementation assays (Figure 5A). The mutants were studied for their ability to restore AmyQ α-

amylase secretion in the secretion-deficient *prsA3* mutant (AmyQ assay) and ability to support growth in cells depleted of the endogenous wt PrsA (viability assay). Immunoblotting of cells producing mutant PrsA proteins revealed that unlike PrsA3 mutant, these mutant proteins were present at levels comparable to wt. No degradation was seen in immunoblotting indicating that mutant proteins are most likely correctly folded and therefore stable. Trypsin accessibility assay carried out with protoplasts of some mutants showed the correct location of the proteins on the outer surface of the membrane.

The deletion mutant without 17 most C-terminal residues forming the so-called serine tail (see section 2.2.4) was fully active in the complementation assays. However, the deletion of half of the C-terminal domain or any deletion extending further from the C-terminus (C-terminus and/or PPIase domain) abolished the activity. A mutant with an N-terminal deletion of 33 amino acid residues was inactive as well. Furthermore, the mutant protein $PrsA_{N+C}$ containing the N- and the C-terminal domains but not the PPIase domain was unable to support growth although it did display a weak activity (15% of the wt) in the AmyQ secretion assay. The integrity of native like fold of $PrsA_{N+C}$ was confirmed by circular dichroism (CD) spectroscopy confirming that the weak or null activity was not due to aggregated or misfolded protein.

Complementation assays with deletion mutants clearly showed that all three domains of PrsA are needed for *in vivo* activity. Only the serine-rich region in the C-terminus could be deleted without any effect on the *in vivo* activity. The role of the middle domain is explained by the enzymatic PPIase activity but the function of the flanking N-and C-terminal domains is unknown. There are several explanations for the role of these domains. They may act as interaction domains to bring the PPIase domain in contact with the substrates in a manner similar to the flanking domains of some PPIases (e.g. Pin1, trigger factor, hPar14, and immunophilins in the Hsp90 complex) by mediating the binding to substrates or cellular components (Radanyi *et al.*, 1994; Lu *et al.*, 1996; Hesterkamp *et al.*, 1997; Reimer *et al.*, 2003). Alternatively, since PrsA seems to be a multimeric protein (R. Seppälä-Lehto, data to be published) the flanking domains might be required for multimerization; in the FK506-binding protein Mip the N-terminal domain is such a dimerization domain (Kohler *et al.*, 2003). Finally, PrsA might also be a modular protein in which domains operate separately in unrelated functions.

Deletion of the PPIase domain abolished the PrsA activity *in vivo*. In contrast to PrsA, PPIases with a deletion of the PPIase domain are functional in *E. coli*. SurA and FkpA devoid of the PPIase domain seem to be functional in the assembly and folding of outer membrane proteins suggesting that their *in vivo* function is

independent of the PPIase domain (Behrens *et al.*, 2001; Saul *et al.*, 2004). However, there are several PPIases in the *E. coli* periplasm (see section 2.2.3), and therefore the possibility of interference by other PPIases in the complementation assays cannot be excluded. Especially, parvulin-type PPIase PpiD may have overlapping activities with SurA since the combination of *surA* and *ppiD* null mutations causes synthetic lethality (Dartigalongue and Raina 1998). In contrast, PrsA is the only extracytoplasmic PPIase in *B. subtilis* and therefore no other PPIases interfere with the assays.

We also characterized PrsA by insertion mutagenesis. Using *in vitro* transposon mutagenesis based on Mu transposition (Poussu *et al.*, 2004), a large set of inframe insertions of five amino acids in the diverse positions of PrsA was obtained. Immunoblotting showed that PrsA levels in the insertion mutants were at the wt level and the signal peptide was cleaved off suggesting translocation across the membrane and by this means correct location. Trypsin accessibility assay of three insertion mutants also indicated the correct location of mutant proteins on the outer surface of the membrane. Very little degradation was seen in immunoblotting indicating the stability of mutant proteins. However, during protein purification many of these mutant proteins turned out to more degradation prone than wt PrsA.

Pentapeptide insertions in the N-terminal domain (residues 1-115) either inactivated PrsA totally or reduced its activity severely. Insertions close to the N-terminus seemed to affect the activity even more severely than the ones close to the parvulin domain. In the parvulin (residues 116-206) and C-terminal (residues 207-273) domains insertions were mostly tolerated. There were only two mutations which were located in the very N-terminus and C-terminus of the PPIase domain, respectively that fully abolished the *in vivo* activity in both complementation assays. However, many mutants having the insertion in the parvulin or C-terminal domain exhibited different activities in the two assays; they complemented the secretion defect of the *prsA3* cells in the AmyQ assay but were unable to fully restore the growth in the viability assay.

In contrast to what was expected, PrsA tolerated insertions well since most insertions did not affect the *in vivo* activity in the complementation assays. This was especially seen in the AmyQ assay. An interesting point is also the fact that many insertion mutants exhibited different activities in the two assays. There are several possibilities for the high tolerance of insertion in the AmyQ assay. In this assay in addition to the overproduced insertion mutant PrsA, there is PrsA3 present in the cells. PrsA3 mutant protein is highly degradation sensitive and therefore its level in cells is low. Yet its *in vivo* activity is similar to the wt (Hyyryläinen *et al.*, 2001), and the decreased AmyQ secretion in *prsA3* cells is simply due to the low amount of

PrsA3. Since it seems that PrsA is a multimeric protein (R. Seppälä-Lehto, data to be published), the insertion mutant PrsA might form a functional complex with PrsA3 protein and therefore restore the AmyQ secretion by intramolecular complementation. Even if the insertion PrsA mutant itself was inactive the multimerization or other interaction between the insertion mutant protein and PrsA3 might stabilize the unstable but otherwise functional PrsA3 and restore AmyQ secretion in this manner. Alternatively, insertion mutant PrsA proteins might interact with quality control proteases and by competitive binding prevent degradation of PrsA3. Insertion mutations in the N-terminus inactivated PrsA. Considering the effect of N-terminal mutations and the possible explanations above allows the hypothesis that mutant proteins with an insertion in the N-terminus were unable to form multimers and thus the N-terminal domain might be responsible for the multimerization of PrsA. In FK506-binding protein Mip the N-terminal domain has been shown to be such a dimerization domain (Kohler *et al.,* 2003).

In the viability assay no intramolecular complementation or other interactions are possible due to the deletion of chromosomal *prsA* gene. Insertion mutant PrsA is solely present in cells and there is no interference of chromosomally encoded PrsA. This difference in settings of complementation assays might explain why some insertion mutants exhibited activity in the AmyQ assay but were inactive in the viability assay. Results of viability assay indicate that actually many insertions in the PPIase and C-terminal domains did have an effect on the activity of PrsA, even though this effect is not seen in the *prsA3* background. Altogether, insertions in all three domains affected the *in vivo* activity confirming that all domains in PrsA are required for the fully functional protein.

5.3 Molecular modeling of the PPIase domain of PrsA (I)

PPIase domain of PrsA was modeled by taking advantage of the known 3D-structure of the PPIase domain of a human parvulin hPar14 (Sekerina *et al.,* 2000; Terada *et al.,* 2001) that is 41% identical to the PPIase domain of PrsA. PPIase domain of PrsA could be modeled into a typical parvulin like fold. According to the model, the amino acid residues predicted to be important in the catalytic site of parvulins (see section 2.2.3.3) were structurally conserved. Both His residues of this site were conserved (His122 and His199 in PrsA), however Asp and Tyr (Asp154 and Tyr196 in PrsA) substituted for Cys and Ser. Yet these replacements are seen in other parvulins as well: in hPar14 and in several bacterial parvulins Cys is replaced by Asp and Ser is also often replaced by other residues (Sekerina *et al.,* 2000). The hydrophobic substrate-binding pocket is formed by Leu, Met and Phe residues in PrsA as in hPar14 and Pin1. Comparison of the surface charge of PrsA and hPar14

revealed that in PrsA the proline-binding pocket and the catalytic site are clearly more hydrophobic than those of hPar14 most likely indicating a difference in substrate specificity. hPar14 is known to have specificity to basic amino acids (Uchida *et al.*, 1999). Despite few differences in active-site residues, both the substrate-binding pocket and the catalytic site are structurally conserved in PrsA and hPar14. It seems that structural conservation can be maintained even with some changes in the amino acid composition (Sekerina *et al.*, 2000; Terada *et al.*, 2001).

5.4 PrsA catalyzes the folding of ribonuclease T1 and *cis/trans* isomerization of peptidyl prolyl bond (I)

PPIase assays *in vitro* were carried out to demonstrate that PrsA indeed has a PPIase activity. First, we examined the ability of PrsA to catalyze the refolding of ribonuclease T1 (RNase T1) (see section 2.2.3). PrsA was purified in the lipomodified form from the membrane by ion exchange chromatography using n-octyl β-D-glucopyranoside as a solubilizing detergent. The secreted, non-lipomodified form of PrsA devoid of the diacylglycerol lipid anchor was generated by replacing the type II signal sequence of *prsA* with the type I signal sequence of *amyQ*, and thus the protein was released into the culture medium instead of the membrane anchoring. The culture medium was concentrated prior to purification of secreted PrsA by ion exchange chromatography. The PPIase activity of both the lipomodified and non-lipomodified PrsA was measured. PrsA catalyzed the refolding of RNase T1 in a concentration dependent manner. The catalytic activity calculated as the k_{cat}/K_m value was 143 mM^{-1} s^{-1} for the lipomodified and 25 mM^{-1} s^{-1} for the non-lipomodified form, respectively.

Next, a set of deletion mutants was constructed and expressed as GST-fusion proteins in *E. coli*. The fusion proteins were purified by affinity chromatography and the GST-part of the fusion protein was cut off during the purification. The activities of the PrsA mutants were then measured in the RNase T1 refolding assay. The activity was dependent on the PPIase domain since $PrsA_{N+C}$ devoid of the PPIase domain was inactive (Figure 5B). Additionally, the PPIase domain alone was sufficient for activity since all mutant PrsA proteins with deletions in the N- and C-terminal domains and even the PPIase domain only catalyzed the refolding of RNase T1 in a similar manner as the full-length PrsA (Figure 5B). Neither CsA nor FK506 inhibited the activities of PrsA mutants indicating that PrsA preparations were not contaminated with cyclophilins or FK506-binding proteins. The results indicate that the PPIase domain indeed forms a separate domain, which is able to fold correctly into active conformation without the influence of the flanking regions. The enzymatic activity of PrsA was low compared to the activity of the positive control

cyclophilin (1300 mM^{-1} s^{-1}) used in the experiments and trigger factor (Stoller *et al.*, 1995) but comparable to the activity of *E. coli* SurA and Parvulin (Scholz *et al.*, 1997b; Behrens *et al.*, 2001)

The PPIase activity *in vitro* was also measured using a protease-coupled PPIase assay and succinyl-Ala-Lys-Pro-Phe-4-nitroanilide tetrapeptide as the substrate (see section 2.2.3). Consistently with the RNase T1 refolding results, wt and the PPIase domain alone ($PrsA_{PPIase}$) exhibited activity while PrsA devoid of the PPIase domain ($PrsA_{N+C}$) was inactive (Figure 5B). Altogether, the activities were low compared to the activity of cyclophilin (900 mM^{-1} s^{-1}), Pin1-type parvulins and *E. coli* Parvulin (Ranganathan *et al.*, 1997; Scholz *et al.*, 1997b) but comparable to the activities of SurA, PpiD and hPar14 parvulins (Dartigalongue and Raina 1998; Uchida *et al.*, 1999; Behrens *et al.*, 2001).

Low PPIase activities of PrsA may be due to non-optimal conditions *in vitro*. PrsA is located on the outer surface of membrane in which the pH is very low (pH 3-4) due to the proton motive force (Kemper *et al.*, 1993). Both PPIase assays were carried out in near neutral pH, which may not be optimal for the activity of PrsA. Moreover, since PrsA is a positively charged protein, interactions with negatively charged cell wall components might be important for its full activity. The fact that the lipomodified PrsA was more active in the RNase T1 refolding experiments than the non-lipomodified form supports the hypothesis that PrsA requires a specific environment and conditions.

5.4.1 Predicted catalytic amino acid residues are dispensable for PrsA function *in vivo*

Next, the importance of the conserved residues locating in the active site of the PrsA PPIase domain was studied. Residues His122, Asp154, Tyr196 and His199 in the catalytic site and Met172 and Phe176 in the substrate-binding pocket were replaced either with alanine or tyrosine. Additionally, five residues (Phe144, Ser152, Gly161, Gly197 and Ile200) were replaced with alanine and Ser156 by proline. These residues are either conserved within the parvulin family (Phe144, Ser152, Gly161) or substitution of corresponding residues (Gly197 and Ile200) in the PpiD parvulin inactivated PpiD both *in vivo* and *in vitro* (Dartigalongue and Raina 1998). Moreover, three internal deletions were constructed: ΔM172-K182 removed most part of the substrate-binding pocket and deletions ΔD154-Q171 and ΔT185-K202 flanked the substrate-binding pocket. In immunoblotting most of the mutant PrsA proteins were present at wt levels suggesting the correct conformation. In the case of

three mutants, $PrsA_{H199}$, $PrsA_{\Delta D154\text{-}Q171}$ and $PrsA_{\Delta T185\text{-}K202}$, partial degradation was detected.

Most active site residues or otherwise conserved residues could be substituted without any effect on the PrsA function *in vivo*. Only few mutants showed some phenotypic effect. The mutations G197A and H199A and the deletion of substrate-binding pocket (ΔM172-K182) resulted in a partial activity in the AmyQ secretion assay (80, 40 and 70% of wt, respectively) whereas the mutation Y176A affected the viability resulting in a partially restored growth in cells depleted of wt PrsA. Since mutation H199A caused a partial degradation of the protein and, since there is a linear relationship between the amount of PrsA and AmyQ secreted (see section 5.7.3) we cannot conclude whether in this case the effect on AmyQ is due to the lowered amount of PrsA mutant or due to the H199A mutation which inactivates the protein. The deletions ΔD154-Q171 and ΔT185-K202 did inactivate PrsA. However, based on the modeling these long deletions most likely disrupt the central β-barrel structure of the PPIase resulting in the loss of 3D-structure. Partial degradation of these deletion proteins supports the hypothesis of incorrect folding. Therefore, no conclusions on the activity of these deletion mutants can be made. To conclude, none of the mutations totally abolished the *in vivo* activity indicating that none of the predicted active site residues is absolutely indispensable for the PrsA function *in vivo* in AmyQ secretion and cell viability.

Despite the high tolerance of mutations in the PPIase domain, the domain is most likely essential for the PrsA function *in vivo* as evidenced by the inability of the PrsA mutant lacking the PPIase domain ($PrsA_{N+C}$) to restore AmyQ secretion or viability. An alternative explanation for the inactivity of $PrsA_{N+C}$ would be that the N- and C-terminal domains are incorrectly folded without the PPIase domain. However, the spectrum CD spectroscopy indicated that $PrsA_{N+C}$ is in correctly folded conformation. Both the native-like fold of CD spectrum of the $PrsA_{N+C}$ and the full enzymatic activity of the PPIase domain alone indicate that the PrsA domains are able to fold correctly independently. The essential nature of the PPIase domain is also supported by the fact that according to the model, the pentapeptide insertions in the PPIase domain that had an effect on the PrsA function were located around the predicted active site. This suggests that despite the high tolerance of mutations in the active center, the PPIase domain is important. However, if the active site can be substituted without effect on the phenotype, the essential *in vivo* activity of PrsA is not the enzymatic peptidyl prolyl isomerization activity. Indeed, in some parvulins the PPIase activity seems to be non-essential. Deletion of the PPIase domain in SurA or FkpA does not affect their function in folding and assembly of outer membrane proteins in the periplasm of *E. coli* (Behrens *et al.*,

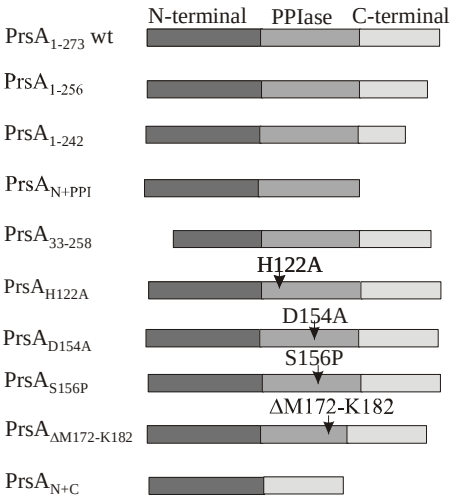
2001; Saul *et al.*, 2004). A PrsA-like protein PmpA of *L. lactis* may also function independently of its PPIase domain (Drouault *et al.*, 2002).

5.4.2 Predicted PPIase active site is important for activity in *vitro*

Several substitution mutants of PrsA were subjected to the RNase T1 refolding and protease-coupled PPIase assays *in vitro* (Figure 5B). Since the PPIase domain alone was sufficient for the full enzymatic activity *in vitro*, the PPIase domain carrying a point mutation S156A, H122A or D154A ($PrsA_{PPI\text{-}S156P}$, $PrsA_{PPI\text{-}H122A}$ and $PrsA_{PPI\text{-}D154A}$) or the deletion the substrate-binding pocket ($PrsA_{PPI\text{-}\Delta M172\text{-}K182}$) was used in the assays. The PPIase domains carrying the mutation were produced as GST-fusions in *E. coli*.

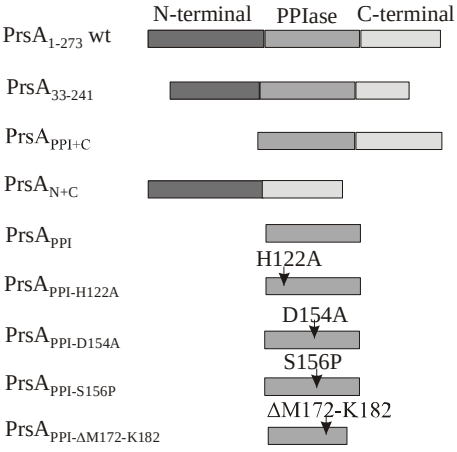
The mutant proteins $PrsA_{PPI\text{-}S156P}$, $PrsA_{PPI\text{-}H122A}$ and $PrsA_{PPI\text{-}D154A}$ catalyzed the RNase T1 refolding only marginally and $PrsA_{PPI\text{-}\Delta M172\text{-}K182}$ was totally inactive. However, in the protease-coupled PPIase assay with a tetrapeptide substrate only $PrsA_{PPI\text{-}H122A}$ and $PrsA_{PPI\Delta M172\text{-}K182}$ were inactive. Although $PrsA_{PPI\text{-}S156P}$ and $PrsA_{PPI\text{-}D154A}$ were clearly defective in the refolding of RNase T1, in the protease-coupled assay both mutants exhibited an activity about half of that of the wt $PrsA_{PPI.}$ This indicates that proline-limited folding of a complex substrate such as RNaseT1 requires some other determinants in the PPIase domain than those required for the catalytic prolyl isomerization activity. This is also the case for SurA, in which the PPIase domain I, though inactive as a prolyl isomerase on its own, provides determinants that allow active PPIase domain II to interact with RNase T1 in such way that RNase T1 becomes a better substrate for the catalysis at the active site of domain II (Behrens *et al.*, 2001). However, these determinants in the PPIase domain of PrsA do not seem to be important for *in vivo* activity since both $PrsA_{PPI\text{-}S156P}$ and $PrsA_{PPI\text{-}D154A}$ are fully active in the complementation assays.

A



	Complementation AmyQ secretion	Complementation Viability
$PrsA_{1-273}$ wt	+++	+++
$PrsA_{1-256}$	+++	+++
$PrsA_{1-242}$	-	-
$PrsA_{N+PPI}$	-	-
$PrsA_{33-258}$	-	-
$PrsA_{H122A}$	+++	+++
$PrsA_{D154A}$	+++	+++
$PrsA_{S156P}$	+++	+++
$PrsA_{\Delta M172-K182}$	70%	+++
$PrsA_{N+C}$	15%	-

B



	Refolding of RNase T1 k_{cat}/K_m (mM/s)	Prolyl isomerization k_{cat}/K_m (mM/s)
$PrsA_{1-273}$ wt	25	6
$PrsA_{33-241}$	22	nd
$PrsA_{PPI+C}$	21	nd
$PrsA_{N+C}$	bd	bd
$PrsA_{PPI}$	28	15
$PrsA_{PPI-H122A}$	weak	bd
$PrsA_{PPI-D154A}$	weak	6
$PrsA_{PPI-S156P}$	weak	8
$PrsA_{PPI-\Delta M172-K182}$	bd	bd

Figure 5. Activity of wt PrsA, deletion mutants and PPIase active-site mutants in vivo and in vitro. Panel A shows the results of complementation assays in vivo. +++, wt activity, - no activity. Panel B shows the in vitro activities in the RNaseT1 refolding assay and in the PPIase assay with tetrapeptides. bd, below detection, nd, not done and weak, activity nearly below detection.

In Pin1 parvulin the residues corresponding to His122 and Asp154 in PrsA are highly important for the PPIase activity *in vitro*. The substitution of these residues in Pin1 resulted in an almost complete loss of the activity measured by the protease-coupled assay (Ranganathan *et al.*, 1997). Consistent with the results in Pin1, the substitutions of the corresponding residues in PrsA affected the PPIase activity: H122A was inactive and D154A decreased the activity to half of that of the wt. Furthermore, deletion of the substrate-binding pocket ΔM172-K182 abolished the PPIase activity totally. The H122A, D154A and ΔM172-K182 mutants were also defective or totally inactive in catalyzing the refolding of RNase T1. These results suggest that the predicted active site residues are indeed crucial for the PPIase activity of PrsA.

In contrast to the results above, the mutations H122A, D154A and ΔM172-K182 placed in the full-length PrsA had wt activity in the RNaseT1 refolding assay. In these mutants the flanking domains in the full-length protein may be able to provide determinants for protein folding by some mechanism other than isomerization of the prolyl bonds. An analogical situation is found in the DnaK chaperone, which in addition to the chaperone function, is able to catalyze *cis/trans* isomerization of nonprolyl bonds thereby promoting protein folding (Schiene-Fischer *et al.*, 2002).

5.5 Exoproteome studies on PrsA depletion and active site mutations (I)

Two-dimensional SDS-PAGE analysis was used to study the effects of partial PrsA depletion and four active site mutations of the PPIase domain on the exoproteome. Chromosomal *prsA* was placed under IPTG inducible P_{spac}-promotor, and the resulting strain was first cultivated with IPTG followed by cultivation without IPTG to the early stationary phase of growth. During the cultivation without IPTG the partial depletion of PrsA did not affect growth yet.

The partial depletion of PrsA caused prominent alterations in the extracellular proteome. Firstly, elevated amounts of cytoplasmic proteins were observed indicating cell lysis. This result is consistent with the cell lysis observed in microscopy in the absence of PrsA (see section 5.1). Secondly, the depletion decreased or abolished most secreted proteins with type I and II signal peptides: these proteins include carbohydrate metabolic enzymes, proteases, nucleotidases, lipases, cell wall hydrolases, phage-related and flagella-related proteins and proteins with unknown functions. However, some proteins with the predicted type I signal peptide, and therefore expected to be translocated by the Sec pathway, were present in levels similar to wt. Results suggest that the stability and secretion of several

exoproteins seems to be dependent on PrsA, but not all. Altogether it is difficult to evaluate how much PrsA depletion actually decreased the secretion of exoproteins due to the cell lysis that caused a high level of contaminating cytoplasmic proteins in the exoproteome.

Four active site mutations studied above (H122A, D154A, Y196A and ΔM172-K182) were introduced in cells with the wt PrsA depletion as plasmid-encoded PrsA proteins and studied whether these mutant proteins were able to restore the normal protein pattern. $PrsA_{H122A}$ restored the wt pattern, which is consistent with the complementation assay result (Figure 5A). It seems that the mechanism of effects on the exoproteome is independent of the PPIase activity since $PrsA_{H122A}$ was able to restore the wt pattern though it was nearly or totally inactive in the RNAse T1 refolding and PPIase assay with tetrapeptides, respectively (Figure 5B). $PrsA_{Y196A}$ also restored the wt pattern despite that there was a moderate defect in the viability assay. However, the exoproteome of ΔM172-K182 and D154A mutants resembled that of the partial depletion of the wt PrsA. There was a decrease in secreted proteins and some cell lysis, yet the amount of cytoplasmic proteins was less than in the partial depletion of wt PrsA. These two active-site mutants seemed to only partially restore the wt pattern indicating that these mutants are to some extent defective even though they fully complemented in the complementation assays.

5.6 PrsA does not possess a general chaperone activity (I)

Some PPIases have an additional chaperone activity (see section 2.2.3.4). Two chaperone assays, the rhodanese and citrate synthetase (CS) assay, were used to study if PrsA is able to function as a molecular chaperone. Both assays are based on the ability of a chaperone to rescue a substrate protein from aggregation during the refolding. In both assays, the substrate is first unfolded with denaturing agent and then refolded in the absence or presence of a chaperone.

In the rhodanese assay, in the absence of a chaperone only 30% of the rhodanese enzymatic activity was recovered by spontaneous refolding. GroEL chaperone, which was used as a positive control, increased the recovery up to 80%. Unlike GroEL, PrsA did not increase the recovery of rhodanese and thus exhibited no chaperone activity. In the citrate synthase assay the formation of aggregation during refolding was measured by light scattering. Similarly to the rhodanese assay, the result was negative: PrsA did not prevent the aggregation of CS.

Both assays are widely used to detect chaperone activity and have been used in the case of other parvulins and PPIases in general (Ideno *et al.*, 2000; Behrens *et al.*,

2001; Pirkl *et al.*, 2001; Ramm and Pluckthun 2001; Ideno *et al.*, 2002). Even though any chaperone activity of PrsA with the common substrates could not been shown, the possibility of a specific chaperone activity with an unidentified substrate is not excluded. As discussed in the case of low PPIase activity (see above), a unique microenvironment might be needed for the chaperone activity as well.

5.7 Capacity of the secretion apparatus for secretion of α-amylase (II)

Expression of exported proteins at a high level causes saturation of the secretion machinery (Leskelä *et al.*, 1999b). To study this saturation of secretion, AmyQ α-amylase was used as a model protein. Cells expressing AmyQ from a xylose-inducible system (P_{xyn}-*amyQ*) were cultivated at different xylose concentrations (0.02, 0.04, 0.08, 0.16 and 0.2% of xylose) to express *amyQ* at different levels. Levels of secreted AmyQ and cell-associated pre-AmyQ and mature AmyQ were then measured. The cell-associated AmyQ is in two forms: the precursor form (pre-AmyQ) with the signal sequence and the mature AmyQ from which the signal sequence is cleaved off.

The saturation of the secretion machinery was seen already at a rather low xylose induction level. The amount of secreted AmyQ leveled off at 0.04% xylose and more induction did not increase the secretion. However, the total amount of AmyQ (secreted and cell-bound) increased up to the highest xylose concentration since the pre-AmyQ in cells increased in a concentration-dependent manner. While at the threshold concentration of 0.04% there was hardly any visible precursor form, at the highest xylose concentration of 0.2%, the majority of the cell bound AmyQ was in the precursor form.

The specific secretion rate at 0.04% xylose induction at several timepoints was determined. The specific secretion rate at the cell density of Klett 100 + 1 h was 10 fg/h/cell after 1 h of induction, the rate decreased over time and was 2.5 fg/h/cell three hours later. During the same time the accumulation of AmyQ in the culture medium increased from 2 μg/ml to 14 μg/ml. At the secretion level of 10 fg/h/cell one cell secretes approximately 30 AmyQ molecules per second. If it is assumed that there are few hundred translocases per cell (see section 5.1), an AmyQ molecule is translocated every 10 second, which is in agreement with the estimated translocation rates of individual precursor proteins in *E. coli* (Randall 1983; Bassilana and Wickner 1993).

5.7.1 PrsA deficiency does not affect the cell-associated accumulation of AmyQ precursors

Previous studies on PrsA have indicated that although PrsA is involved in the posttranslocational stage of secretion, it is not involved in the translocation event itself (Jacobs *et al.*, 1993). This data was verified by studying the pattern and location of the pre-AmyQ in cells depleted of PrsA. Cells with the inducible P_{spac}-*prsA* in the chromosome and the xylose-inducible *amyQ* expressed from a plasmid were cultivated in the presence of several xylose concentrations. Simultaneously, P_{spac}-*prsA* was either fully induced with 1 mM IPTG or partially induced with 24 μM IPTG. The IPTG concentration of 24 μM was chosen as the concentration for partial induction of *prsA* because it still supported a growth rate similar to that of wt (see section 5.1). The amounts of PrsA and secreted and cell-associated AmyQ were determined by immunoblotting. At 1 mM induction, *prsA* was expressed at the full induction level and an abundant band of PrsA was detected in cell samples. At this induction level, the threshold for saturation of AmyQ secretion was already at the starting concentration of 0.02% xylose. Pre-AmyQ was detected in cells already at this concentration and it accumulated in a xylose concentration-dependent manner. Due to the saturation of the secretion machinery already at the lowest xylose concentration used, the amount of AmyQ in the culture medium was same in all concentrations studied. At 24 μM IPTG a weak band of PrsA was detectable in cell samples. As expected, very little AmyQ was accumulated in the culture medium. Yet, at this low PrsA level the amount and pattern of cell-associated AmyQ were quite similar to the one with the full induction. This demonstrates that PrsA depletion did not affect the accumulation of precursors in the cells, and thus PrsA is not involved in the translocation process across the cytoplasmic membrane or in the processing of the exported proteins.

The cellular location of accumulated pre-AmyQ was studied by a trypsin accessibility assay. Both *prsA3* mutant cells overexpressing AmyQ and its wt parent were grown in the presence of 0.02% xylose to induce *amyQ*. In the trypsin accessibility assay protoplasts were prepared followed by incubation with trypsin in the presence or absence of a membrane solubilizing detergent Triton-100. Levels of pre-AmyQ, PrsA and GroEL were measured by immunoblotting. GroEL was used as a trypsin-sensitive cytoplasmic protein to monitor the lysis of protoplasts during the assay. There was hardly any degradation of GroEL detected indicating that the protoplasts remained intact. As expected, the level of PrsA3 protein was only 10% of the wt level and yet the same portion of pre-AmyQ (70%) was degraded by trypsin treatment in both wt and *prsA3* strains. This 70% of pre-AmyQ was thus primarily exposed on the outer surface of the protoplasts and susceptible to trypsin

while the remaining 30% of pre-AmyQ was likely inside the protoplasts and not yet translocated. No AmyQ was detected when protoplasts were treated with Triton X-100 to lyse the cells prior to the trypsin digestion. Since the same portion of pre-AmyQ was exposed on the outer surface of protoplasts in *prsA3* mutant than in the wt cells, the PrsA deficiency did not increase the level of intracellular pre-AmyQ.

Both saturation threshold experiment and trypsin accessibility experiment clearly show that PrsA protein is neither involved in the translocation nor in the processing of secreted proteins. Neither the threshold level of saturation nor the pattern of cell-associated AmyQ was affected by PrsA depletion. These results are consistent with the pulse-labeling experiments in which the PrsA deficiency did not affect the rate of pre-AmyQ processing (Leskelä *et al.,* 1999a). Although there was no difference in the pattern of cell-associated AmyQ, the decreased amount of secreted AmyQ is seen in PrsA depleted cells. This indicates that posttranslocational folding is impaired in cells depleted of PrsA resulting in degradation of secreted proteins after their translocation.

5.7.2 Rate of signal peptide processing limits the secretion of AmyQ

Since PrsA deficiency did not affect the saturation of secretion machinery, the possible role of a signal peptidase (SP) as a limiting factor was tested. SipT is known to be the main SP responsible for the processing of pre-AmyQ though there are overlapping substrate specificities in SPs (Tjalsma *et al.,* 1997). The *sipT* gene was overexpressed from a plasmid and the same P_{xyn}-*amyQ* construction as above was used for controlled expression of *amyQ*. Again, cells were cultivated at different xylose concentrations and precursor and mature forms of AmyQ in the cells were detected by immunoblotting. In cells overexpressing *sipT* the threshold for the saturation of pre-AmyQ processing was higher (0.16% of xylose) than in wild type cells (0.08% of xylose). Moreover, the portion of mature AmyQ in cells was higher in the SipT overproducer than in wt, demonstrating that the signal peptidase is a rate-limiting factor for AmyQ secretion. Yet, the enhanced processing of pre-AmyQ did not increase the amount of secreted AmyQ in the medium, indicating that there are still additional limiting factor such as PrsA. Previous studies have shown that the SPs are indeed limiting factors for protein secretion of some exoproteins (Bron *et al.,* 1998). The substrate preference of SPs is still quite unknown (Bolhuis *et al.,* 1996; Bron *et al.,* 1998), which means that to improve secretion, an optimal combination of SPs has to be sought for each exoprotein.

5.7.3 PrsA is needed for the secretion of α-amylase expressed at high and low level

To relate the rate of AmyQ secretion to the number of PrsA molecules in cells, two strains with controlled expression of *prsA*, at a high and low level, were constructed. In the first strain, IH7163, *prsA* was placed under the P_{spac} promotor in the chromosome; with this construction low levels of PrsA were obtained. Even with full induction the level was only 40% of the P_{prsA}-*prsA* level suggesting that P_{spac} was a weaker promotor than the native one. The native *prsA* gene in the chromosome was deleted. In the same strain, AmyQ was expressed constitutively from a plasmid at the rate saturating the secretion machinery. IH7163 cells were cultivated at different IPTG concentrations (0-100 μM) and samples were withdrawn at the early stationary phase of growth. The cell density was determined by counting the cells by microscopy. Quantitation of PrsA in cell fraction was determined using purified PrsA protein as standard in immunoblotting. Accumulated AmyQ in the culture medium was measured by enzymatic assay. PrsA levels were found to increase as a function of IPTG concentration, and simultaneously, the amount of AmyQ in the culture medium increased as well. At the highest IPTG concentrations tested (100 μM) PrsA level did not increase any further, probably because maximum induction had already been reached. The results indicate that in the conditions where the PrsA level is the limiting factor for AmyQ secretion there was a linear correlation between the number of PrsA molecules in cells and the number of AmyQ molecules in the culture medium. This finding is consistent with the previous studies (Kontinen and Sarvas 1993) pointing out that PrsA is a bottleneck for the secretion of exoproteins expressed at a high level.

To examine whether the dependency of AmyQ secretion on the PrsA level was simply due to the liming amount of PrsA, we studied the secretion of AmyQ in conditions reversed to the IH7163 strain. In the strain 7162 *prsA* was overexpressed from P_{spac}-*prsA* from a plasmid and *amy*Q was expressed from a single chromosomal copy at a low level below the saturation point. At 100 μM IPTG induction the PrsA level was ten-fold higher than that of wt and twenty fold higher than in the strain IH7163 above. Still, the dependency of AmyQ secretion on the level of PrsA was seen and although the dependency was not completely linear, not even at the highest amount of PrsA studied (about 200 000 molecules per cell), the secretion of AmyQ was not leveling off. Thus PrsA may be beneficial not only when proteins are secreted at high level but also at low levels of expression. Several biotechnically important proteins are secreted in amounts below the levels that saturate the secretion machinery, and thus the overproduction of PrsA may increase the yield of these proteins as well. Especially important PrsA might be in the

production of such valuable proteins whose expression is kept low to ensure correct folding and minimize degradation.

The dependence of AmyQ secretion on PrsA even in conditions of huge excess of the folding factor compared to the secreted protein suggests reversible association and disassociation reactions between PrsA and an exoprotein. This type of action is typical to chaperones, which undergo several reaction cycles with the substrate until folding is complete (see section 2.2.1.1). The dependency might also indicate that only a fraction of PrsA molecules are available at a time. Therefore the number of PrsA molecules near by the translocase complex could be a limiting factor in spite of the great number of PrsA proteins in general. Such an excess of protein may be an advantage for cells in some growth conditions. An example of such a case is DivIB cell division protein of *B. subtilis*: much higher levels of DivIB are needed for normal growth when the temperature is elevated from 30°C to 47°C (Rowland *et al.*, 1997). Many proteins involved in protein folding are highly expressed in stress conditions (Rosen and Ron 2002). However, PrsA is an abundant protein already under normal growth conditions and its expression is not induced under stress conditions such as heat or secretion stress (H.-L. Hyyryläinen, data to be published).

5.8 Some heterologous proteins are PrsA-dependent on their secretion in *B. subtilis* (III)

The exoproteome studies above show that many exoproteins in *B. subtilis* are dependent on PrsA. Moreover, the results above show a linear dependency between PrsA and AmyQ model protein. Thus PrsA as a folding factor rises hopes for its utility in biotechnical applications; does the engineering of PrsA level offer a means to improve the secretion of heterologous proteins? To elaborate this, the effect of PrsA on the secretion of 11 bacterial exoproteins of biotechnical interest was studied. Most of these heterologous proteins were expressed from a *Bacillus* secretion vector from the *amyQ* promotor and translocated by the aid of the *amyQ* signal sequence (Palva 1982). Previous studies have shown that using the expression system above proteins are secreted in *B. subtilis* with very different efficiencies: yields varied from a few g/l to less than 1 mg/l of protein in the culture medium. This is most likely due to the posttranslational events and indeed, proteolytic degradation of some model proteins after their translocation has been shown to be the cause of low yields in some cases (Ulmanen *et al.*, 1985).

First, the PrsA-dependency of model proteins was studied (Table 3). The same P_{spac}-*prsA* construction as in the strain IH7163 above was used. With this construction the PrsA level without any induction was enough for normal growth due to the leakiness

of P_{spac}-promotor. The strain was transformed with plasmids carrying genes encoding heterologous proteins and cells were grown with or without 1 mM IPTG. To determine the yields of heterologous proteins, samples were withdrawn at several time points for enzymatic assays and at the early stationary phase of growth for immunoblotting. In most cases both culture medium and cells were analyzed.

The α-amylase of *B. stearothermophilus* (AmyS) was found to be a strongly PrsA-dependent protein. The PrsA depletion decreased its accumulation in the culture medium to about 10% of the non-depleted level. Another PrsA dependent protein of Gram-positive bacteria was pneumolysin (Pnl), an antigen of *Streptococcus pneumoniae* with diagnostic value; PrsA-depleted cells secreted only about 30% of the non-depleted level. The PrsA level also affected the amount of cell-associated Pnl since more protein was detected in non-depleted than in PrsA depleted cells. The secretion of two bacillar enzymes, β-glucanase (BglA) and β-lactamase (BlaP) of *B. licheniformis*, was only slightly reduced in PrsA depleted cells suggesting that these enzymes are PrsA-independent. Another PrsA-independent protein was diphteria toxoid (DT) of *Corynebacterium diphtheriae*, which is a potential target drug for cancer cells. Furthermore, PrsA had a slightly negative role on plasminogen activator staphylokinase of *S. aureus* since its secretion was 20% higher in cells of depleted of PrsA than in the non-depleted ones.

Secretion of some proteins of Gram-negative bacteria was PrsA-dependent as well. Pectin hydrolyzing enzyme endopolygalacturonase (Peh) of *Erwinia* spp. was shown to be strongly PrsA-dependent, its secretion decreased to about 30% by the depletion with a concomitant decrease in the amount of the cell-associated Peh. In contrast, pectin methylesterase (Pme) of *Erwinia* spp. was independent of PrsA. Another PrsA-dependent protein was TEM-1 β-lactamase (TEM-1) of *E. coli*, its secretion decreased into half in PrsA depleted cells. PrsA depletion had no effect on the secretion of pertussis toxin subunits S1 and S4 of *B. pertussis* but depletion did reduce the cell-associated form of S4. Altogether, the secretion of four heterologous proteins was shown to be dependent on PrsA, thus demonstrating a tool of engineering which could be used to gain more productive yield of heterologous proteins in *B. subtilis*.

5.8.1 PrsA overproduction may be beneficial or deleterious on the secretion depending on the exoprotein

Next, the effect of PrsA overproduction on the secretion of PrsA-dependent proteins (AmyS, Pnl, Peh and TEM-1) was studied (Table 3). PrsA was overproduced from pKTH277 plasmid resulting in 5-fold increase in PrsA level compared to that of wt

(Kontinen and Sarvas 1993). The protein secretion was determined in the absence and presence of pKTH277. The results above showed that the secretion of AmyS was strongly PrsA-dependent and thus PrsA overproduction did enhance its secretion 4-fold. The enhancement is very similar to the secretion of AmyQ described in section 5.7.3 and in earlier studies on AmyQ and AmyL (Kontinen and Sarvas 1993 and section 2.2.4.2). These bacillar α-amylases are the most strongly PrsA-dependent proteins. Results suggest that α-amylases and α-amylase-like proteins are good targets when PrsA technology is used to enhance extracellular protein production and increased protein yields can be expected by co-overproduction of PrsA with α-amylases in industrial processes. In addition to AmyS, Pnl exhibited both PrsA-dependent secretion in the depletion experiments and enhanced secretion (1.5 fold) when PrsA was overproduced, although the increase was less than with α-amylases. This result suggests that PrsA engineering can also be used to enhance yields of other heterologous exoproteins apart from α-amylases. It has been previously shown that PrsA overproduction increased the secretion of a heterologous subtilisin, *B. licheniformis* SubC, in *B. subtilis* about 2-fold (Kontinen and Sarvas 1993) and that the secretion of recombinant *B. anthracis* protective antigen (rPA) in *B. subtilis* is dependent on PrsA (Williams *et al.,* 2003). Furthermore, PrsA overproduction increases the total yield of a secretory single-chain antibody fragment and its secretion in *B. subtilis* (Wu *et al.,* 1998).

In contrast to what was expected, PrsA overproduction did not enhance the secretion of all PrsA dependent proteins and the overproduction was even deleterious for some proteins. Though TEM-1 was PrsA-dependent, its secretion was not affected by PrsA overproduction. The results on Peh were unexpected as well. Though secretion of Peh was clearly PrsA-dependent, the overproduction of PrsA decreased the amount of Peh in the culture medium to 20-30% of that of wt. A possible explanation for this decrease is that PrsA overproduction might increase the secretion of unidentified PrsA-dependent proteases resulting in increased proteolysis and Peh might be more sensitive to proteolysis than some other proteins studied. To overcome this possibility, protease inhibitors were added to the culture medium during growth but this addition did not improve the yield. The inhibitory effect of PrsA was observed also with pertussis subunit S1 though this protein was secreted in a PrsA-independent manner. The native S1 of 28 kDa is proteolytically truncated into 20 kDa form in *B. subtilis* and both forms of S1 are detected in cells. However, the truncated S1 is the major secreted form in *B. subtilis* (Himanen *et al.,* 1991). PrsA overproduction increased the amount of cell-associated forms of S1 and more degradation products were detected than in the wt level of PrsA. In the culture medium, the accumulation of the 20 kDa S1 form decreased to about 30 % of the wt level. The harmful effect of PrsA may indicate that the molar ratio of PrsA to heterologous protein at the membrane-cell wall interface is important for optimal

protein folding and secretion. Altogether, our results indicate that the production of only a subset of heterologous proteins benefit from increasing the level of PrsA.

PPIase activity of PrsA does not explain the effect on heterologous proteins. Firstly, mutagenesis studies indicate that the PPIase activity is non-essential for the *in vivo* function to promote secretion (see section 5.4.1). Secondly, the 3D-structures of AmyL and AmyS do not contain *cis*-prolines (Machius *et al.,* 1995; Suvd *et al.,* 2001) and still these proteins are strongly PrsA-dependent. However, during protein folding the isomerization from *trans* to *cis* and back to *trans* may occur before the final isomeric form of the prolyl bond takes place (Schmid 2001). Therefore, intermediates of proteins might need the assistance of a PPIase although in the final protein conformation all prolyl bonds are in the *trans* form.

5.9 Increased negative net charge of the cell wall effects secretion of some exoproteins (III)

Increased negative charge of the cell wall caused by the *dlt* mutation (see section 2.3.2) improves secretion of some model proteins in *B. subtilis* (Hyyryläinen *et al.,* 2000; Thwaite *et al.,* 2002; Craynest *et al.,* 2003). To explore whether *dlt* mutation could be a general means to enhance secretion, the effect of *dlt* mutation on the heterologous proteins described above was tested (Table 3). Secretion of heterologous proteins was compared between the *dltD* mutant and its wt parent strain. Pnl was the only protein to benefit from the increased negative charge of the cell wall, its secretion enhanced about 1.5-fold. The *dlt* mutation had no effect on AmyS, BlaP, BglA, Pme or DT. Surprisingly, the *dlt* mutation had a harmful effect on secretion of three exoproteins: secretion of pertussis toxin subunit S1 and TEM-1 was decreased by 50% and secretion of PehA by 30%, respectively.

Since only one protein was secreted better in the *dlt* mutant than in wt it seems that the modulating of the negative charge in the cell wall improves yields of a fairly limited number of exoproteins. Enhanced secretion of heterologous cyclodextrin glycosyltransferase in an industrial *B. licheniformis* strain due to a *dlt* mutation has been reported (Craynest et al., 2003). Previous studies also show that secretion of chimeric α-amylases and the protective antigen (PA) of *B. anthracis* produced in *B. subtilis* is increased by a *dlt* mutation (Hyyryläinen *et al.,* 2000; Thwaite *et al.,* 2002). Chimeric α-amylases and PA are secreted at a fairly low level due to considerable proteolytic degradation and at least in the case of chimeric α-amylases the degradation is mainly cell wall-associated (Stephenson and Harwood 1998). The sensitivity to proteolytic degradation in the membrane-cell wall matrix may be one

element that determines the direction of the *dlt* effect on secretion. Sensitivity to proteolysis is not the only factor as can be seen by the decreasing effect of the *dlt* mutation on the secretion of TEM-1. TEM-1 is an extensively degraded protein but the degradation takes place in the culture medium, not in the cell wall (Ulmanen *et al.*, 1985). Secretion of S1 was also impaired in the *dlt* mutant most probably due to decreased conversion of the 28 kDa S1 to the secretable 20 kDa S1. We can conclude from our results that proteins that are degraded only moderately or not at all during secretion such as AmyS, BlaP, BglA and Pme are secreted independently of *dlt*.

Since both the PrsA overproduction and *dlt* mutation enhanced the Pnl secretion it was studied whether combining these beneficial elements migth have an additive effect on the secretion of Pnl. However, the combination did not further increase the Pnl secretion, suggesting that both PrsA overproduction and *dlt* mutation may affect the same folding or degradation step. This fits with the data that the *dlt* mutation rescued PrsA3 from degradation and partially suppressed the PrsA depletion (Hyyryläinen et al., 2000 and section 2.2.4.2).

5.10 Depletion of HtrA-type serine proteases causes growth defects and does not improve the yield of heterologous proteins (III)

HtrA quality control proteases degrade misfolded proteins at the membrane-cell wall interface. The effect of depletion of Htr-type proteases on the secretion of heterologous proteins was tested by disrupting the *cssR* gene. The *cssR* gene encodes the regulator component of the CssSR two-component system, which regulates the expression of *htrA* and *htrB* (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). A previous study by Hyyryläinen *et al.*, (2001) showed that in the *cssS* mutant the expression of *amyQ* at a high level is lethal suggesting that the accumulation of misfolded proteins is very harmful in the absence of HtrA-type proteases. However, when *amyQ* was expressed at low level, the inactivation of the CssSR system moderately increased AmyQ secretion (1.3-fold) compared to wt. These results suggested that depletion of quality control proteases might improve secretion.

A fragment of *cssR* was cloned into pMUTIN4 plasmid and the resulting plasmid was integrated into the chromosome disrupting the *cssR* gene. Plasmids encoding heterologous proteins described above were then introduced into the *cssR* mutant and secretion of these proteins was compared between the mutants and the respective wt parent strains (Table 3). In contrast to the results by Hyyryläinen *et al.*,

(2001), though heterologous proteins were expressed at low levels harmful effects were observed in the *cssR* mutant. Although the expression of *amyS* and *amyL* was low, no beneficial effects on secretion were observed. On the contrary, there were retarded growth and decreased secretion of both α-amylases. The production of Pnl had even more severe effect on growth. Stable *cssR* mutants were obtained only at a low temperature (20°C) and even at this temperature their growth was retarded and secretion impaired. Production of Pme in the *cssR* mutant was harmful on growth in a similar temperature-dependent manner as that of Pnl. Yet, Pme was secreted at the wt level. In contrast to other model proteins studied in the *cssR* mutant, BlaP and Peh were secreted at the wt level without any effect on growth. Less misfolding may occur during secretion of these two proteins than of the other proteins and their folding may be less dependent on chaperones and other folding factors. Since efficient folding reduces accumulation of misfolded proteins and the need for quality control proteases, normal growth and secretion in the *cssR* mutant is possible. Altogether, these results indicate that the presence of HtrA-type cleaning proteases is not a limiting factor for protein secretion. On the contrary, in many cases these proteases are a necessity for cells to survive. These results lead to conclusion that engineering of HtrA-type proteases may not be a feasible means to improve secretion of heterologous proteins in *B. subtilis*.

Table 3. Effects of PrsA, *dlt* mutation and depletion of HtrA proteases on secretion of heterologous proteins studied.

Protein	PrsA dependency	PrsA overproduction	*dlt* mutation	Depletion of HtrA proteases
AmyS	yes(+)	↑	no effect	↓
BglA	no		no effect	
BlaP	no		no effect	no effect
Pnl	yes(+)	↑	↑	↓
DT	no		no effect	
Sak	yes(-)			
Peh	yes(+)	↓	↓	no effect
Pme	no		no effect	no effect
TEM-1	yes(+)	no effect	↓	
S1	no	↓	↓	
S4	no			

yes(+), positive dependency, yes(-) negative dependency, ↑, increase in secretion and ↓, decrease in secretion.

5.10.1 HtrA-type proteases are responsible for the processing of pertussis toxin subunit S1

Depletion of HtrA proteases affected the secreted and cell-associated form of pertussis toxin subunit S1. The major secreted form of S1, the 20 kDa degradation product (Himanen *et al.*, 1991), accumulated into the culture medium in reduced levels. At the logarithmic phase of growth, no 20 kDa S1 was detected in the culture medium. At the stationary phase of growth, the protein was detected but even then its amount was only 5% of that of the wt level. In cells, there was hardly any detectable 20 kDa form present at any growth phase, only the non-truncated 28 kDa form of S1 was seen indicating that virtually no processing took place when HtrA proteases were down regulated in the *cssR* mutant. This result suggests that HtrA and/or HtrB proteases are responsible for the processing of the 28 kDa S1 to the 20 kDa S1 consequently secreted into the medium. To identify the protease responsible for the processing, *htrA* and *htrB* genes were deleted separately. Both deletion of *htrA* or *htrB* caused some decrease in the S1 processing but the effect was much milder than in the *cssR* mutant in which both *htrA* and *htrB* are down regulated. In the culture medium of *htrA* and *htrB* mutants the level of the secreted 20 kDa form was slightly decreased and there was increased accumulation of the 28 kDa form in cells. These results suggest overlapping activities for HtrA and HtrB in the processing of pertussis toxin S1.

It is unclear why the intact 28 kDa form of S1 is not secreted in *B. subtilis.* In *B. pertussis* the secretion of pertussis toxin involves the association of the S1 subunit to the outer membrane, and this targeting requires a characteristic outer membrane motif at the C-terminus of S1 (Farizo *et al.*, 2002). Whether the C-terminal motif functions as a stop transfer signal in *B. subtilis* and thereby would anchor the 28 kDa form to the cytoplasmic membrane and cause its retention was not studied. Alternatively, the 28 kDa S1 may be tightly associated with the cell wall polymers or other components in the cell envelope. Possibly HtrA proteases recognize the C-terminally anchored 28 kDa S1 in the membrane or cell wall as a misfolded/misplaced protein and cut it off releasing most of the protein as the 20 kDa S1 into the medium. The knowledge about the substrate specificity of HtrA proteases is limited. However, studies with *E. coli* DegP indicate that the proteolysis occurs between hydrophobic residues (Jones *et al.*, 2002).

6 CONCLUDING REMARKS

Efficient protein secretion is a sum of several cellular factors and processes. The interests of this study were the late stages in protein secretion, namely the posttranslocational folding of proteins. This folding takes place after the translocation of proteins across the cytoplasmic membrane and is affected by several components in the membrane-cell wall environment. The focus was on the role of the PrsA lipoprotein. Exoproteome studies showed that PrsA is required for the secretion of a substantial number of exoproteins, and thus PrsA is a necessary component of the secretory pathway in *B. subtilis*. It was indisputably showed that PrsA is essential for viability of *B. subtilis* cells, and cell morphology gave clear clues about what might be its essential role. The specific role of PrsA in the folding of proteins or enzymes required for cell wall synthesis needs to be verified. An interesting question is also why PrsA is such an abundant protein on the cell membrane. This abundance suggests a chaperone-like function for PrsA though chaperone assays *in vitro* did not detect such an activity. Yet, nowadays the word chaperone is often used in a broad sense meaning all proteins promoting folding of proteins and PrsA certainly fits into this definition.

This study greatly contributes to the understanding of PrsA lipoprotein as a component of secretory pathway and protein folding in *B. subtilis*. An enzymatic peptidyl prolyl *cis/trans* isomerase activity of PrsA was determined *in vitro*, which allowed us to categorize PrsA into the class of parvulin-type PPIases. Earlier this classification had been based on sequence similarities only. However, the *in vivo* activities still remain unclear since extensive mutagenesis analysis revealed that PPIase activity is not the *in vivo* function of PrsA in the conditions studied. All domains of PrsA are clearly needed for the functional protein and mysterious non-PPIase activity. It is yet unclear whether the domains are functioning separately or contributing to the same activity. Structural studies of the protein will certainly reveal new information about PrsA.

The capacity of secretion machinery no doubt sets limits for protein secretion. This study clearly concluded that PrsA is not involved in the translocation events itself though it is a limiting factor for secretion of some exoproteins. PrsA also enhanced secretion of a PrsA-dependent exoprotein in condition where there was a huge excess of PrsA compared to the exoprotein. Again, this kind of action suggests a chaperone-like function for the protein.

Development of biotechnical processes for production of valuable native and heterologous proteins is one of the main goals in research of secretion machinery of

B. subtilis. This requires engineering of components in the secretory pathway. PrsA overproduction enhanced the secretion of some model proteins and is a good candidate to be tested when optimising protein secretion in *B. subtilis*. Modulation of the properties of cell wall, in this case the net charge of cell wall, positively affected secretion of some exoproteins. The positive effect of PrsA overproduction and increased negative charge of cell wall by *dlt* mutation on individual proteins has been shown by others as well. In this study a larger number of secretory proteins were used as model protein, which allowed making more general conclusions on how the secretion of heterologous proteins can be improved. The data indicates that both PrsA and *dlt* mutation have roles in biotechnological applications. Instead the modulation of the level of the quality control proteases did not improve secretion. On the contrary, the cells suffered from the depletion of HtrA-type cleaning proteases. It seems that the cleaning proteases are vital for cells to survive and therefore the depletion of these proteases cannot be used to improve secretion.

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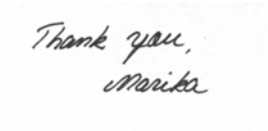

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