Review

Post-translocational folding of secretory proteins in Gram-positive bacteria

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Abstract

The transport of proteins from their site of synthesis in the cytoplasm to their functional location is an essential characteristic of all living cells. In Gram-positive bacteria the majority of proteins that are translocated across the cytoplasmic membrane are delivered to the membrane–cell wall interface in an essentially unfolded form. They must then be folded into their native configuration in an environment that is dominated by a high density of immobilised negative charge—in essence an ion exchange resin. It is essential to the viability of the cell that these proteins do not block the translocation machinery in the membrane, form illegitimate interactions with the cell wall or, through intermolecular interactions, form insoluble aggregates. Native Gram-positive proteins therefore have intrinsic folding characteristics that facilitate their rapid folding, and this is assisted by a variety of folding factors, including enzymes, peptides and metal ions. Despite these intrinsic and extrinsic factors, secretory proteins do misfold, particularly if the cell is subjected to certain types of stress. Consequently, Gram-positive bacteria such as Bacillus subtilis encode membrane- and cell wall-associated proteases that act as a quality control machine, clearing misfolded or otherwise aberrant proteins from the translocase and the cell wall.

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1. Introduction

All living cells are subdivided into distinct compartments, most of which are confined by membranes. As protein synthesis takes place primarily in the cytoplasm, proteins that are functional in other compartments need to be transported from the cytoplasm to their destination compartment. The cell-associated compartments that can be distinguished in Gram-positive bacteria such as Bacillus subtilis are the cytoplasm, the cytoplasmic membrane and the cell wall. Additionally, a significant number of proteins are secreted into the environment. In recent years, genomic and proteomic tools have been employed to generate an inventory of the so-called secretome of B. subtilis [1,2]. By definition, the secretome includes both the various pathways for the transport of proteins across the cytoplasmic membrane to the membrane–cell wall interface, the cell wall or the extracellular environment, and the secretory proteins themselves. At least five distinct pathways for protein transport have been identified in B. subtilis. The majority of ~300 potentially secretory proteins appear to be translocated by the “Sec” pathway (Fig. 1) for protein secretion [3–5]. Typical proteins of this type include degradative enzymes (e.g. carbohydrases, DNAses, lipases, phosphatases, proteases and RNAses), proteins involved in cell wall biogenesis, substrate binding proteins, and even pheromones involved in sensing the cell population density for onset of developmental processes such as natural competence and sporulation. However, the precise function of a large proportion of proteins that were shown or predicted to follow the Sec pathway remains to be determined. Other pathways for protein transport, such as the twin-arginine translocation “Tat” pathway, a pseudopilin export (Com) pathway involved in natural competence development, phage-like holins, and certain ATP-binding cassette (ABC)
transporters, are “special-purpose” transporters, limited to the export of a small number of specific proteins.

While the Tat pathway has the potential to transport fully folded proteins [6], the Sec pathway exclusively transports proteins in an unfolded or loosely folded state [7]. Upon exit from the channel of the Sec translocase, proteins must fold rapidly into their native conformation. This is not only required for the activity of exported proteins but also for their stability, since the membrane–cell wall interface is a highly proteolytic environment that monitors and maintains...
the quality of secreted proteins [8–12]. As the vast majority of secretory proteins are exported from the cytoplasm by the Sec-dependent translocase, the mechanisms of post-translocational protein folding have attracted considerable interest. The analysis of these mechanisms is of scientific relevance because of unique characteristics of Gram-positive cell envelopes that are not found in their Gram-negative counterparts. Additionally, post-translocational protein folding is of major biotechnological importance in view of the widespread use of Bacillus species for the biomanufacture of proteins and enzymes. The present review is, therefore, focused on the determinants for post-translocational protein folding in B. subtilis (listed in Table 1). Specifically, relevant structural and physico-chemical aspects of the cell wall, extracytoplasmic catalysts for protein folding and the cellular response to extracytoplasmic protein unfolding stress will be addressed. Where appropriate, similarities or differences with related mechanisms in other bacteria are highlighted.

2. Structure of the cell envelope

The environment into which secretory proteins of Gram-positive bacteria emerge from the secretory translocase on the trans side of the cytoplasmic membrane plays an important role in their ultimate fate (Fig. 1). The Gram-positive cell envelope consists of a cytoplasmic membrane and heteropolymeric cell wall (Fig. 2) [13,14]. Peripheral to the cell wall, some strains have proteinaceous surface layers (S-layers) and/or polysaccharide or polypeptide capsules. The edible B. subtilis var. natto, used in Japan for soybean fermentations, secretes copious amounts of the highly viscose flavour-enhancing poly-glutamic acid, while B. anthracis encodes the same polymer as part of its armoury of immune-evading virulence factors [15].

The most notable difference between Gram-negative and Gram-positive bacteria is the absence, in the latter, of an outer membrane and associated membrane-bounded periplasm. The periplasm of Gram-negative bacteria is a dynamic and metabolically highly active environment, encapsulating a variety of enzymes and proteins. Gram-positive bacteria compensate for the lack of a membrane enclosed periplasm by lipo-modifying homologues of many of the proteins located in the periplasm of Gram-negative bacteria (e.g. peptidyl-prolyl-isomerases and transporter substrate binding proteins etc.), anchoring them to the outer surface of the cytoplasmic membrane. Additionally, Gram-positive bacteria selectively immobilise proteins in the cell wall by ionic or covalent interactions. Thus the combination of extensive lipo-modification and the presence of a thick, h

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**Table 1**

<table>
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<tr>
<th>Protein Function</th>
<th>Homologues</th>
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<tr>
<td>Quality control proteases</td>
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<tr>
<td>Membrane associated</td>
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<tr>
<td>HtrA (YkdA) Membrane-associated and extracellular serine protease</td>
<td>DegP of E. coli; HtrA-like proteins in L. lactis and S. mutans</td>
</tr>
<tr>
<td>HtrB (YvtA) Membrane-associated serine protease</td>
<td>DegP of E. coli; HtrA-like proteins in L. lactis and S. mutans</td>
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<tr>
<td>Wall associated</td>
<td></td>
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<tr>
<td>CWBP52 Cell wall-associated serine protease</td>
<td></td>
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<tr>
<td>Folding factors</td>
<td></td>
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<tr>
<td>PrsA Putative peptidyl prolyl cis–trans isomerase</td>
<td>PrsA of L. monocytogenes; PrsAA, PrsAB, PrsAC of B. anthracis; PrtM, PmpA of L. lactis</td>
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<tr>
<td>Propeptides</td>
<td>Intramolecular chaperone or foldase</td>
</tr>
<tr>
<td>Metal cations, e.g. Ca2+, Mg2+, Fe3+</td>
<td>Folding and stability factors</td>
</tr>
<tr>
<td>BdhA (YolI) Extracytoplasmic thioldisulfide oxidoreductase</td>
<td>Bdb of B. brevis</td>
</tr>
<tr>
<td>BdhB (YolK) Membrane-associated thioldisulfide oxidoreductase involved in sublancin 168 production</td>
<td>DsbB of E. coli</td>
</tr>
<tr>
<td>BdhC (YvgU) Membrane-associated thioldisulfide oxidoreductase involved in pseudopilin ComGC production; folding catalyst for S–S bond containing secretory proteins</td>
<td>DsbB of E. coli</td>
</tr>
<tr>
<td>BdhD (YvgV) Extracytoplasmic thioldisulfide oxidoreductase involved in pseudopilin ComGC production; folding catalyst for S–S bond containing secretory proteins</td>
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<tr>
<td>Quality control regulators</td>
<td></td>
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<tr>
<td>CssR (YvqA) Response regulator</td>
<td>CpxR of E. coli</td>
</tr>
<tr>
<td>CssS (YvqB) Sensor kinase</td>
<td>CpxA of E. coli</td>
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Fig. 2. Electron micrograph of a cross section through B. subtilis. (a) heteropolymeric cell wall; (b) cell wall in the processes of being turned over and released by autolysins; (c) cytoplasmic membrane.
negatively-charged cell wall provides Gram-positive bacteria with the means of immobilising specific activities in an extracytoplasmic but cell-associated location [16], in effect a non-membrane-bound periplasm.

2.1. Cytoplasmic membrane

The cytoplasmic membrane of *B. subtilis* is a bilayered structure consisting of phospho- and glycolipids, the relative amounts of which vary in response to the growth conditions [17–20]. Phosphatidylglycerol and phosphatidylethanolamine are the main phospholipids, while diglucosyl-diglyceride, monoglucosyl-diglyceride and 1,2-diglyceride are the main glycolipids. The fluidity of the membrane is adjusted according to the growth temperature. At 37°C phospho- and glycolipids are almost exclusively composed of saturated fatty acids. However, at lower temperatures, membrane fluidity is maintained by increasing the proportion of low-melting-point fatty acids by de novo synthesis of anteiso-branched fatty acids and enzymatic desaturation of existing fatty acids [21,22].

2.2. Cell wall

The cell walls of Gram-positive bacteria are highly cross-linked semi-porous structures that are tightly apposed to the cytoplasmic membrane. They represent dynamically flexible structures that are up to 20 times thicker than the peptidoglycan layer of Gram-negative bacteria [13]. The cell wall is involved in many cellular processes including the maintenance of cell shape, protection of the underlying protoplast, cell division and interactions between the cell and its environment. All ions and molecules entering or leaving the cell must therefore traverse and/or interact with the cell wall. However, the physico-chemical properties of the wall, particularly its limited porosity and high concentration of immobilised negatively charged groups, restrict the movement of compounds (including proteins) between the cytoplasmic membrane and surrounding environment. The wall is also an attachment site for cations involved in the maintenance of an optimum ionic environment for membrane-bound enzymes and the folding of secretory proteins. In addition, the wall is the final destination of specific proteins such as autolysins and other wall-associated proteins (WAPs) [13,14].

The cell walls of Gram-positive bacteria are composed of two types of polymers, namely peptidoglycan (Fig. 3) and covalently attached anionic polymers (usually, teichoic and teichuronic acids—see Figs. 4 and 5), both of which are essential for growth and viability. The cell wall may also contain significant amounts of lipoteichoic acid (LTA) (Fig. 4) and protein. Peptidoglycan, which typically accounts for ~40% of the Gram-positive cell wall, is a heteropolymer consisting of rigid glycan chains, cross-linked with flexible peptide substituents (Fig. 3A) [23]. In *Staphylococcus aureus* the stem peptides are cross-linked with pentaglycine bridges that also have a role in the covalent attachment of wall proteins (Fig. 3B). Cross-linking produces a strong but elastic multilayered net-like structure that surrounds the entire cytoplasmic membrane [13,14].

Anionic polymers, which represent about 50% of the wall by weight, are covalently bound to the glycan chain of peptidoglycan. Phosphate groups in the repeating units of teichoic acids (Fig. 4) and carboxyl groups in the uronic acid residues in teichuronic acids (Fig. 5) are responsible for the majority of negative charge at the surface of Gram-positive bacteria. Electrostatic repulsion between these charged groups is a major contributor to the physico-chemical environment of the cell wall, and has important roles in cell morphogenesis and growth [24,25]. Conditional mutants of *B. subtilis* with decreased amounts of anionic polymers in their cell walls exhibit an irregular coccal cell morphology [26,27] while mutants that are completely devoid of anionic polymer are not viable.

The main teichoic acid of *B. subtilis* strain 168 is a 1,3-linked polyglycerol phosphate in which the hydroxyl group...
at position C-2 of the of the glycerol moiety can have α-glucoyl or α-alanyl ester substituents [28,29]. The positively charged amino group of an alanyl residue can neutralise the negative charge of an adjacent phosphoryl residue, thereby reducing the overall charge of the wall. Strain 168 also synthesises a secondary teichoic acid, namely poly-glucosyl N-acetylgalactosamine 1-phosphate [30,31], while strain W23 has a polyribitol phosphate teichoic acid.

Teichuronic acid is an alternative, non-phosphorus-containing negatively charged polymer that is synthesised under phosphate-limitation in place of teichoic acids in most strains of B. subtilis and in strains of B. licheniformis as a coexisting polymer under most conditions. While the structure of teichuronic acid has not been determined in B. subtilis strain 168, that of W23 consists of repeating units N-acetylgalactosamine and N-acetylglucuronic acid (Fig. 5).

Strains of B. subtilis contain significant amounts (1% to 2% by dry cell weight) of LTA. This polymer is mainly found in association with the cytoplasmic membrane, although a partially deacylated form is also found within the wall, at the cell surface and in the culture supernatant. LTA is composed of chains of polyglycerol phosphate that are attached through their terminal phosphate to 3-gentiobiosyl diglyceride [32], which is responsible for anchoring the molecule to the outer surface of the cytoplasmic membrane. LTAs, like wall teichoic acids, bind bivalent cations and may help to buffer ion concentration at the surface of the cytoplasmic membrane (Fig. 4B) [33]. A second proposed role of LTAs is to bind cell wall-degrading enzymes (autolysins), thereby inhibiting their activity at the wall/membrane interface.

Intact LTA molecules have amphipathic properties due to the presence of the hydrophobic glycolipid and the hydrophilic teichoic acid chain. α-Alanyl substituents have a considerable influence upon the ionic properties of the polymer and, possibly, upon the extent to which it is able to participate in the biosynthesis and assembly of the wall [29].

2.3. Wall-associated proteins

B. subtilis 168 contains a number of WAPs, many of which are autolysins [14,34]. These proteins interact with the wall by strong ionic interactions, and are only removed by high salt concentrations (5 M NaCl) or boiling with detergents such as SDS. These ionically bound proteins often contain repeated domains that are responsible for their interaction with the wall [35]. In other Gram-positive bacterial species, notably S. aureus, a proportion of the WAPs are covalently bound to the cell wall [36] by the sortase enzyme. The C-terminal regions of these proteins have a conserved domain that includes an LPXTG motif followed by a sequence of hydrophobic amino acids (15–19 residues) and then a positively charged region (five to nine residues). Although it encodes a sortase homologue and
proteins with an LPXTG motif, currently there is no evidence for the covalent linkage of any proteins to the wall of *B. subtilis*.

One of the major groups of WAPs comprises the peptidoglycan-hydrolyzing autolysins which are involved in cell wall growth and turnover, cell division, competence and flagellation [13,14]. The major autolysins are N-acetylmuramoyl-l-alanine amidase, encoded by *lytC* [37], and *N*-acetylmuraminidase, encoded by *lytD* [38].

Some WAPs are proteolytically processed following translocation across the cytoplasmic membrane. WapA, a wall-associated protein of unknown function, is synthesised as a 258-kDa precursor that is subsequently processed into wall-bound forms of 220, 109 and 58 kDa [35]. However, a proportion of the 220-kDa form is secreted into the culture supernatant by an as yet unknown mechanism. Similarly, proteins CWBP52 and CWBP23 are the processed products of the *wprA* gene, the primary product of which is a 96-kDa protein [39]. CWBP52 is a wall-bound serine protease involved in the degradation of nonnative secretory proteins [12].

### 3. Folding catalysts

#### 3.1. The PrsA protein

The vectorial transfer of proteins through the translocase implies that secretory proteins must undergo post-translational folding at the outer surface of the cytoplasmic membrane, not unlike the folding of nascent polypeptides emerging from ribosomes. The latter event is assisted and catalysed by several cytoplasmic chaperones and foldases. Similarly, various general or protein-specific chaperones and foldases (e.g. PpiA, PpiD, F6pA, Fkp, SurA and PapD) have been found to be involved in the folding of periplasmic or outer membrane proteins in Gram-negative bacteria. It is interesting, especially in the context of the unique microenvironment at the cytoplasmic membrane–wall interface (see above), that relatively few proteins have been shown to assist the post-translocational folding of Gram-positive bacterial secretory proteins. One such protein, found ubiquitously in all Gram-positive species but not at all in any Gram-negative species, is PrsA.

The PrsA of *B. subtilis* is a membrane-associated lipoprotein of 270 amino acid residues [40,41]. It is a hydrophilic protein, with no predicted membrane spanning regions and a calculated pI of about 10 [40]. It is accessible to trypsin in protoplasts [42] and anchored to the outer surface of the cytoplasmic membrane by the two fatty acid moieties attached to its N-terminal cysteine, in a manner typical of bacterial lipoproteins [42]. PrsA is an abundant protein with about 20,000 molecules per cell and thus in obviously high excess over the number of translocase complexes [43]. The role of the fatty acid modification is to tether PrsA to the outer surface of the membrane. However, the activity of PrsA is retained when it is devoid of fatty acids, but anchored to the membrane by the uncleaved signal peptide. This has been shown for mutants defective in either the lipid modification of lipoproteins [42] or the processing of lipoprotein signal peptides [44].

The biological activity of PrsA was initially addressed through mutation studies [45,46]. Point mutations in the *prsA* gene [45], or depletion of the protein by placing the *prsA* gene under the control of an inducible promoter [43], resulted in decreased secretion of a heterologous model protein, the α-amylase (AmyQ) of *B. amylophiliquefaciens*, expressed at high level in *B. subtilis*. There was a linear relationship between the rate of secretion and the cellular level of the PrsA protein [43]. More recent proteomic studies have demonstrated decreased amounts of several endogenous exoproteins in the growth medium of bacteria partially depleted of the PrsA protein [47]. In contrast, the opposite effect was observed when PrsA was overproduced by expressing the *prsA* gene from a multicopy plasmid or a strong inducible promoter; dramatic increases in the production of heterologous model proteins such as AmyQ or the SubC protease of *B. licheniformis* indicated that the level of PrsA is a potential bottleneck for secretion [41]. This finding indicates approaches to exploiting the *prsA* gene for the removal of crucial bottlenecks in industrial applications (see also the article by Westers et al. in this special issue). Importantly, PrsA can also be exploited to increase the yields of heterologous proteins produced by *B. subtilis*, as exemplified for the protective antigen (rPA) of *B. anthracis* [48]. Severalfold increases in the amounts of rPA in the growth medium were obtained by increasing the expression of *prsA*, not unlike the secretion of AmyQ described above. However, similar effects have not yet been demonstrated in the case of homologous exoproteins expressed from their own promoters.

Several lines of evidence indicate that PrsA is involved in the secretion at a late, post-translational stage. In mutant *prsA* strains or those depleted of PrsA, there is no accumulation of cell-bound non-processed precursors of α-amylase. Furthermore, changes in the cellular levels of PrsA (increases or decreases) have no effects on protein translocation across the membrane nor on the capacity of the translocase [43]. In contrast, pulse-chase labelling showed that a subtilisin-alkaline phosphatase fusion protein was extensively degraded and lost its enzymatic activity in a *prsA* mutant with decreased levels of the PrsA protein [46]. These data support the view that PrsA functions as a chaperone or foldase that facilitates the post-translocational folding of secreted proteins. Reduced levels of PrsA either retard the rate of substrate protein folding or result in an increase in their misfolding; both outcomes result in an increased rate of degradation by proteases located in the wall or at the membrane–cell wall interface [49]. The role of PrsA in the secretion of some heterologous proteins from *B. subtilis* is even more to the point. Single-chain antibodies (SCAs) have been shown to be secreted from *B. subtilis* [50–52],
although a large proportion remained cell-associated and insoluble [51]. Increasing the level of PrsA rendered that fraction largely soluble, and increased the amount that was secreted into the culture medium. It appears that scarcity of PrsA resulted in the aggregation of misfolded SCAs, although the cellular location of the precursor forms was not determined in this study. On the other hand, the secretion of another SCA was only slightly improved in the presence of high levels of PrsA in a similar system [52]. Interestingly, secretion of this SCA was enhanced and degradation decreased by eliminating the major cell wall protease, WprA. The latter finding is indicative of the inherent problem of post-translocational folding of heterologous proteins, and the prevention of their subsequent proteolytic degradation, that can only partly be overcome by increasing the production of PrsA. These studies further demonstrate the potential of manipulating prsA expression in the biotechnological production of various types of proteins of interest.

PrsA of B. subtilis is an indispensable protein. Depletion of the number of PrsA molecules below ca. 500 copies/cell retards growth, causes gross morphological alterations and eventually results in cell death and lysis [43]. The distorted cell morphology of PrsA-depleted cells resembles that of cells in which the synthesis of cell wall polymers is inhibited [13]. This suggests that PrsA assists the folding of at least one essential extracytoplasmic protein, or the extracytoplasmic domain of an integral membrane protein, involved in the synthesis of the wall matrix.

Putative proteins sharing extensive amino acid sequence similarity with the PrsA protein of B. subtilis are encoded by the genomes of all Gram-positive bacteria sequenced to date (for a recent survey, see Ref. [53]). Some of these proteins have been characterised. Three potential PrsA proteins (PrsAA, PrsAB, PrsAC) have been identified in B. anthracis [48]. When expressed in B. subtilis, all of these proteins complemented the function of B. subtilis PrsA [48]. The B. subtilis genome encodes a parologue of PrsA, namely YacD. However, YacD is not essential for growth nor, apparently, for secretion, and its function remains unknown (Ref. [48], and Sarvas et al., unpublished results).

Two prsA homologues have been characterised in several lactococci [54–56]. In Lactococcus lactis the homologue PrtM is required for the production of active PrtP, a membrane-bound protease with a large extracytoplasmic domain, in a manner consistent with a role as a folding catalyst. The second PrsA-like lipoprotein of L. lactis is PmpA [53]. PmpA is not involved in PrtP maturation, but has been shown to stabilize some secreted heterologous proteins [57].

3.2. Similarities between PrsA-like proteins and peptidyl-prolyl cis/trans-isomerases

The detailed molecular mechanism by which PrsA-like proteins facilitate the folding of secreted proteins remains to be elucidated. A crucial clue to their activity, however, may be their sequence similarity to prokaryotic and eukaryotic peptidyl-prolyl cis/trans isomerases (PPIases) of the parvulin family [58–60]. All PrsAs are modular proteins, in which the middle domains share sequence similarity with the PPIase domains of parvulins [59]. N-terminal domains of PrsA proteins, typically about 120 amino acid residues in length, share sequence similarity only with PrsA proteins of closely related Gram-positive bacteria such as Bacillus and Listeria species; there are hardly any similarities between the C-terminal domains of PrsA proteins from different species.

PPIases are ubiquitous proteins, and their activity is crucial for rapid folding of proteins with cis-prolyl residues [58]. The function of PrsA-like proteins in enhancing the folding of secreted proteins is in good agreement with PPIase activity. In this context, it is intriguing that the trigger factor, which assists the folding of nascent polypeptides emerging from ribosomes, is also a PPIase [61]. Additionally, some of the periplasmic folding factors associated with Gram-negative bacteria, such as PpiA, FbpA and the parvulin-like proteins PpiD and SurA [62], also display PPIase enzymatic activity in vitro. The in vivo role of the PPIase activity and even that of the PPIase domain of any secretion related PPIase homologue, however, remains controversial. The best-studied example is the periplasmic SurA protein of Escherichia coli, which assists in the folding and assembly of outer membrane proteins. Mutant SurA proteins, which were inactive in in vitro PPIase assays, or even devoid of the active parvulin domain, fully complemented surA null mutations in vivo. Such mutant proteins, however, showed chaperone activity in vitro. The authors concluded that the main function of SurA is that of a chaperone, an activity mediated by the N- and C-terminal domains of this protein. In contrast, the precise role of the central PPIase domain remains unclear. The structure of SurA, as determined by X-ray crystallography, appears to be in agreement with a dual functionality. The PPIase domain is a distinct module linked to the core module at some distance [63,64]. Additionally, as observed by Drouault et al. [53], the PPIase domains of PrsA-like proteins from streptococci and lactococci differ by several residues from the consensus signature of the PPIase motif, although one of them, PmpA of L. lactis, clearly participates in the folding of a secreted protein. Furthermore, various mutant B. subtilis PrsA proteins with short insertions in the PPIase domain, and a mutant PrsA protein that is not active in the in vitro PPIase assay, still supported growth and, when overproduced, enhanced the secretion of AmyQ [47,65]. Taken together, these results suggest that the PPIase activity is of minor importance for the function of PrsA in vivo, which would be counter-intuitive in view of the invariable presence of PPIase domains in all PrsA-like proteins [53].

It is possible that conditions of in vitro PPIase assays deviate significantly from those at the membrane–wall interface and are thus misleading, or that as yet unidentified
secreted and cell-associated AmyQ in B. subtilis, there appears to be no such effect on AmyQ synthesised by protoplasts. Furthermore, the folding of AmyQ, as determined by protease sensitivity, took place at comparable rates irrespective of the amounts of PrsA that were present. This suggests that the need for PrsA is associated with some feature of the microenvironment at the membrane–wall interface, which is consistent with the observed rapid and unassisted folding of AmyQ in vitro [66].

3.3. Thiol-disulfide oxidoreductases

Disulfide bonds are essential for the activity and stability of numerous eubacterial and eukaryotic proteins. In principle, disulfide bonds can be formed spontaneously in vitro, but this process is much slower and less effective than the formation of disulfide bonds in vivo, where it is catalysed by thiol-disulfide oxidoreductases [67,68]. In Gram-negative bacteria, such as E. coli, the formation of disulfide bonds occurs mainly in the periplasm, which is a relatively oxidising cellular compartment compared to the cytoplasm. At least six E. coli proteins have been identified that are involved in disulfide bond formation in proteins exported from the cytoplasm [69]. These are known as DsbA, DsbB, DsbC, DsbD, DsbE and DsbG. DsbA is the major oxidase for disulfide bond formation in the periplasm, while the inner membrane protein DsbB is required to maintain the oxidised state of DsbA. The DsbC protein acts as an isomerase that allows “proof-reading” of newly formed disulfide bonds. For this purpose, DsbC is kept reduced by the integral membrane protein DsbD. DsbE is a reductase involved in the biogenesis of cytochrome c, while DsbG seems to be a DsbC-like isomerase. E. coli strains lacking functional dsb genes display pleiotropic phenotypes, including defects in the correct formation of two intramolecular disulfide bonds in the alkaline phosphatase PhoA. Importantly, these two disulfide bonds are required for the activity and stability of PhoA [70].

In contrast to Gram-negative bacteria, relatively little is known about the catalysis of disulfide bond formation in extracytoplasmic or secreted proteins of Gram-positive bacteria. So far, only three extracytoplasmic proteins with disulfide bonds have been identified in B. subtilis. Two of these are the pseudopilins ComGC and ComGG, which are essential for DNA uptake by competent cells. While ComGC molecules contain one intramolecular disulfide bond, an intermolecular disulfide bond can be formed between two ComGG molecules [71]. The third protein is the lantibiotic sublancin 168 (SunA), which contains two disulfide bonds [72]. Notably, engineered disulfide bonds were shown to be formed readily in the secreted neutral protease NprE of B. subtilis [73]. Also, when E. coli PhoA is produced and secreted by B. subtilis, as judged by its activity and stability, its two disulfide bonds are formed [74].

The first extracytoplasmic thiol-disulfide oxidoreductase to be identified in a Gram-positive bacterium was Bdb (Bacillus subtilis disulfide bond formation) from Bacillus brevis [75]. This protein, which has a predicted N-terminal signal peptide, can complement an E. coli dsbA mutant. Unfortunately, the physiological role of Bdb in B. brevis has not been documented thus far. The presence of extracytoplasmic thiol-disulfide oxidoreductases in B. subtilis became evident as a result of the sequencing of the genome of this organism [76]. Database searches aimed at identifying thiol-disulfide oxidoreductases of B. subtilis revealed the presence of three extracytoplasmic enzymes of this type, which were named BdbA (YoiI), BdbB (YolK) and BdbC (YygU) [77]. A fourth extracytoplasmic thiol-disulfide oxidoreductase, named BdbD (YygV), was identified in the course of the Bacillus subtilis Systematic gene Function Analysis (BSFA) programme [78]. BdbA (137 residues) and BdbD (222 residues) are synthesised with predicted N-terminal signal peptides [1], while BdbB (148 residues) and BdbC (138 residues) are typical membrane proteins with four predicted transmembrane segments, their N- and C-termini being localised in the cytoplasm [77]. Interestingly, BdbB and BdbC are paralogous proteins showing significant sequence similarity with DsbB of E. coli. BdbD shares sequence similarity with the DsbA proteins of a variety of Gram-negative bacteria and S. aureus [78].

The bdbA and bdbB genes are located immediately downstream of the sunA gene for sublancin 168 and the sunT gene that encodes an ABC transporter required for sublancin 168 processing and export [79]. This raised the question as to whether BdbA and BdbB might be involved in the production of sublancin 168. While BdbA was shown to be dispensable for this process, BdbB turned out to be of major importance for sublancin 168 production [79]. In the absence of BdbB, only small amounts of sublancin 168 were produced. This residual sublancin 168 production was not observed in a bdbB bdbC double mutant strain, even though the bdbC single mutation had no apparent influence on sublancin 168 production. These observations indicate that BdbC can partly replace BdbB in sublancin 168 production. Although indirect effects of BdbB and BdbC in the process of sublancin 168 production cannot be ruled out completely, it seems most likely that the formation of the two disulfide bonds in this lantibiotic is catalysed by BdbB and, to a minor extent, by BdbC [79].

BdbC and BdbD were shown to be required for the biogenesis of the pseudopilin ComGC, which is consistent with the observation that natural competence development in bdbC or bdbD mutant strains is severely reduced [77,78]. In contrast, disruption of bdbA or bdbB had no detectable effect on competence development [77]. Furthermore, BdbC and BdbD turned out to be of major importance for the post-translocational folding of E. coli PhoA when expressed in B. subtilis [77,78]. BdbB was shown to have a minor role in the latter process, while BdbA seems not to be involved [77]. Although it appears highly likely that BdbC and BdbD
are required to catalyse disulfide bond formation in both *B. subtilis* ComGC and *E. coli* PhoA, it has not been possible to demonstrate this directly. This is due to the fact that both exported proteins are highly sensitive to proteolysis in the absence of BdbC or BdbD.

Interestingly, recent studies have shown that BdbC and BdbD can interfere with the covalent attachment of heme to apocytochrome *c* [80,81]. This relates to the fact that c-type cytochromes of *B. subtilis* are membrane-bound proteins that expose their heme-binding domains at the extracytoplasmic side of the membrane. Heme is covalently attached to apocytochrome *c* through two thioether bonds, a prerequisite of which is that the cysteine residues in the heme-binding site remain reduced. Two membrane-associated thiol-disulfide oxidoreductases have been implicated in the reduction of apocytochrome *c*. These are CcdA, which displays sequence similarity to DsbD of *E. coli* [82], and ResA. Mutant strains that either lack CcdA [81], or are depleted of ResA [80], are unable to form cytochrome *c*. Under these conditions, the biogenesis of cytochrome *c* can be restored by mutations in *bdbC* or *bdbD* [80,81]. This implies that BdbC and BdbD catalyse disulfide bond formation in the heme binding sites of apocytochrome *c* molecules if these are not reduced by CcdA and ResA, most likely through the transfer of reducing equivalents from the cytoplasm.

Taken together, the above observations show that the substrate specificities of BdbB and BdbC with respect to sublancin 168, *B. subtilis* ComGC and *E. coli* PhoA overlap only partly, despite the fact that these typical thiol-disulfide oxidoreductases are highly similar paralogues. As judged by their function in the folding of exported proteins with disulfide bonds, it has been proposed that BdbB, BdbC and BdbD primarily act as oxidases. This is supported by the fact that BdbB and BdbD share a high degree of similarity with DsbB of *E. coli*, while BdbD shares some similarity with DsbA of *E. coli* [77,78]. Finally, it is noteworthy that the composition of the extracellular proteomes of *bdbA*, *bdbB*, *bdbC* or *bdbD* single mutant strains, or a mutant lacking all four of these genes, was indistinguishable from that of the extracellular proteome of the parental strain 168 (H. Antelmann, personal communication). This suggests that, apart from sublancin 168, very few secretory proteins of *B. subtilis* contain disulfide bonds that are indispensable for their stability and protease resistance.

### 3.4. Propeptides of secreted proteins

Cleavable propeptides of various length and locations in the precursors of some secreted proteins represent significant folding factors. They are predominantly found at the N-terminal end of the primary translation product, between a typical signal peptide (prepeptide) and the N-terminus of the mature protein. Propeptides do not have a role in protein translocation across the membrane, which is initiated by the signal peptide, but they are essential in the post-translocational folding process to achieve the active and stable form of the secretory protein. Concomitantly with promoting folding, active propeptides are absolutely required for the passage of such proteins through the bacterial cell wall. Typically, but not exclusively, propeptides are found in inactive zymogens or precursors of secreted proteases. Inactive precursors are a feature shared universally by proteases in all types of cells (for a review, see Ref. [83]). For studies on the function of propeptides, bacterial serine proteases have served as the most important model proteins. These include the α-lytic protease of the Gram-negative bacterium *Lysobacter enzymogenes* and subtilisins secreted by *Bacillus* species. Although these proteases are very different, their propeptides share essential features [83–85].

In general, the propeptides of serine proteases serve two functions. First, they are necessary for the rapid folding of these enzymes through a molten globule-like intermediate state. Under native conditions, propeptides behave as “intramolecular chaperones” or foldases. However, they are also fully active in promoting folding when synthesised as separate molecular entities [86,87]. In the absence of their propeptide, serine proteases do not fold properly and are, generally, not secreted by the bacterial cell. Upon denaturation in vitro, mature proteases can refold into their active conformation if the propeptide is provided in trans. If the propeptide is absent during refolding, these proteins form stable, molten globule-like folding intermediates. The propeptide binds to the corresponding preprotein and guides or catalyses its rapid folding by stabilising the secondary structure at the site of binding, thereby generating a nucleus for folding [88]. Propeptides of proteases from widely different sources seem to adopt similar structural scaffolds although they share little or no sequence similarity, the actual sequences possibly reflecting adaptations to the membrane–wall microenvironments of different cell types [87]. Second, propeptides inhibit proteolytic activity. They bind to the active site of an enzyme and cap it stereochemically. The final step in the folding of a protease is a self-cleavage reaction in which the propeptide is removed autocatalytically by the pro-enzyme. Degradation of the propeptide locks the enzyme in a stable and protease-resistant conformation, facilitating the release of an enzymatically active and presumably secretion-competent protease molecule. Consequently, active-site mutations not only interfere with the proteolytic activity of subtilisin, but also with the process of folding and maturation.

Propeptide-dependent folding and secretion of proteases in *B. subtilis* have been shown to be linked. Early studies [86,89–91] revealed that mutationally inactivated proteases or those devoid of a propeptide were not secreted; inactive precursor was retained in the cell, but could be released to the growth medium by simultaneous production of active subtilisin from an intact gene [89]. In addition, the secretion of the propeptide and the mature parts as separate molecules resulted in the secretion of active, mature-sized subtilisin. A similar pattern has emerged from studies of other Gram-
positive bacteria [91–93]. Thus, it would appear that folding to a compact mature form and removal of the propeptide are necessary for most proteases to traverse the cell wall. However, there are also exceptions. In Streptomyces a proteolytically inactive protease can be secreted with the propeptide attached [94], and extracellular maturation [95] through proteolytic cleavage by another protease was also observed. In general, the influence of the propeptide on protease folding kinetics in the context of membrane and cell wall passage, the subcellular location of folding intermediates and possible accessory factors involved, still remains to be elucidated in detail.

Remarkably, very few studies have addressed the possible requirement for and role of additional folding factors in the secretion of proteases. They are, however, likely to be present. For example, self-maturation of the lactococcal PrtP protease is dependent on PrtM [96], and overproduction of the PrsA protein also enhances the secretion of a heterologous subtilisin in B. subtilis [41].

Lipases of both Gram-positive and Gram-negative species form a second well-characterised family of proteins secreted with a propeptide [97]. The prototype Gram-positive lipase is that of S. hyicus [57]. Like typical protease precursors, its precursor is comprised of three domains: an N-terminal signal peptide, a long propeptide and a region corresponding to the mature lipase. Following signal peptide cleavage, the protein is released into the culture medium as an enzymatically active form, where the propeptide is proteolytically removed by a metalloprotease [98, 99]. This pattern of cleavage has been established not only for other staphylocoecal lipases [100], but also for the autolysin, lysostaphin [101]. Again, mutant studies indicate that the lipase propeptide plays a role both in secretion and its post-translocational stability. Deletions in the propeptide decrease the rate of secretion and result in the production of an inactive protein [99]; no in vitro folding studies have been published. The staphylocoecal lipase is secreted poorly by the non-staphylocoecal hosts L. lactis and B. subtilis, with evidence of degradation. This may indicate the presence of as yet unidentified lipase-specific folding factors in S. hyicus [57]. In this respect, it is interesting to note that secreted lipases of Gram-negative bacteria require specific chaperones for their assembly in the periplasm [102, 103].

The propeptide of the S. hyicus lipase displays an unexpected and very general capacity to prevent the degradation of secreted heterologous proteins unrelated to lipases. A proof-of-principle was the demonstration that the signal peptide and propeptide of the S. hyicus lipase direct the secretion of E. coli β-lactamase in S. carnosus [104]. Without the propeptide there was very little secretion, but cellular accumulation of enzymatically active β-lactamase. The same principle has since been applied to the secretion of many other proteins, such as immunoglobulins [105] and human growth hormone [92]. Furthermore, the S. hyicus lipase propeptide protects the E. coli outer membrane protein, OmpA, against degradation. When expressed in Staphylococcus carnosus or B. subtilis, OmpA is mainly found in cell-associated and insoluble forms, with only degradation products detectable in the culture medium [106–108]. This pattern of production was dramatically altered by extending the N-terminus of OmpA with the propeptide of S. hyicus lipase [108], which significantly increased amounts of OmpA in the culture medium with little or no evidence of degradation products. The absence of degradation reflects the physiological function of the propeptide. The authors concluded that the propeptide facilitates the release of unfolded proteins from the translocase and/or passage through the cell wall, protecting them from proteases at the membrane–cell wall interface. A very different conclusion was drawn from studies by Sturmfels et al. [92]. Fusion of various staphylocoecal propeptides to human growth hormone seemed to maintain the proprotein in a secretion competent form in the cytoplasm before engagement with the translocase.

In some secreted proteins the signal peptide cleavage site is followed by a short stretch of amino acids not found in the secreted, mature exoenzyme. These include, for example, the α-amylase AmyE of B. subtilis [109] and staphylocoecal nuclease [110]. However, these “propeptides” are dispensable for the secretion, folding and stability of the respective mature proteins. Moreover, they are removed nonspecifically in the culture supernatant by proteolytic nibbling.

3.5. Divalent cations and components of the cell wall as folding catalysts

Secreted proteins exit the secretory translocase into a microenvironment which, due to the high density of negative charge associated with anionic polymers, is rich in metal cations, particularly Ca2+, Fe3+ and Mg2+. The metal ions affect both the folding and stability of proteins in this environment. A comparable situation is found in the endoplasmic reticulum of eukaryotic cells, where folding of some, but not all, proteins is modulated through local variations in the Ca2+ concentration [111, 112]. Although the divalent cations are mainly adsorbed to the matrix of the cell wall, they are also readily available to interact with proteins [113].

Many exoproteins contain Ca2+ binding sites or bind Ca2+ [114]. Notably, these include Bacillus subtilisin-like proteases [83, 114]. The role of Ca2+ in the stability of these enzymes has been clearly demonstrated in vitro studies showing that Ca2+ has no role in the early steps of the folding [83, 115] but contributes to the stabilisation of the mature protein following the release of the propeptide. Unfortunately, there are no in vivo studies relating the stabilisation of the folding state to the secretion and translocation of subtilisins through the cell wall.

The contribution of metal ions to the post-translocational folding and secretion of proteins in B. subtilis has been extensively elucidated in studies with the model protein
levansucrase. Early studies demonstrated a cell-associated form of the size of the secreted protein, which was slowly released from the cell in an Fe³⁺-dependent manner [116]. A correlation was observed between a dependence on Fe³⁺ and Ca²⁺, and the rate of in vitro refolding after denaturation by urea or EDTA. In the absence of cationic ions, refolding was arrested at an intermediate stage; the native-like conformation was reached by addition of Fe³⁺ or Ca²⁺ [117,118]. Furthermore, at pH 5.8, both the release in vivo and renaturation in vitro were more efficient and less dependent on the Ca²⁺ concentration. Consistent with these findings, a mutation in the Ca²⁺ binding site of levansucrase decreased the rate of secretion, and the in vitro refolding of the mutant protein required a 10-fold higher concentration of Ca²⁺ than that of the wild type [113,119]. A similar role for Ca²⁺ was observed in the secretion and folding of a chimeric amylase, a derivative of the α-amylase of B. licheniformis (AmyL) engineered for a higher positive charge and pI of 10.0. [120]. Although native AmyL is degraded in the cell wall of B. subtilis, the engineered AmyL was considerably more susceptible to cell-associated proteolysis, consistent with its in vitro refolding becoming more Ca²⁺-dependent. Additionally, the engineered AmyL bound Ca²⁺ with lower affinity than the native AmyL.

Modulation of the folding of translocated proteins by direct interaction with anionic polymers alone or complexed with Ca²⁺ in the cell wall could also be anticipated. The high charge density of the wall might be an additional factor that restricts the physico-chemical properties, in terms of their charge or pI, of proteins that can traverse the cell wall. Indeed, a number of studies point to such interactions. The teichoic acid analogue, polyphosphate [121], inhibits the folding of α-amylases (AmyE of B. subtilis or a mutant AmyL of B. licheniformis), but enhances the folding of levansucrase in a Ca²⁺-independent manner [121]. The in vitro folding of the above chimeric α-amylase, unlike that of AmyL, was retarded by isolated cell wall fragments in a manner suggesting irreversible binding to anionic polymers [120]. Additionally, phosphate depletion, expected to affect the composition of the anionic polymers, enhances the secretion of various exoproteins [122].

The availability of metal cations to act as folding effectors depends on the overall net charge of the cell wall and is partly modulated by the extent to which teichoic acid is N-D-alanylated. When the B. subtilis dlt operon, responsible for the alanylation of teichoic acids, was inactivated, there was a significant increase in the production of a number of secretory proteins, including the amylases from B. amyloliquefaciens (AmyQ) and B. licheniformis (AmyL) and B. anthracis protective antigen (PA) [123,124]. The increase in negative charge resulting from the absence of α-alanylation, particularly in the region of the membrane–wall interface, is likely to increase the wall’s affinity for cations such as Ca²⁺. This would increase the availability of such cations for post-translational folding, either from solution or by their transfer from ligand to ligand (i.e. from teichoic acid to protein) without the need for intermediate solvation.

Taken together, a number of findings support the involvement of metal ions and metal ion–polymer complexes in the post-translational folding of exoproteins and their release through the cell wall. However, any conclusions should be drawn with caution. The cell wall is a very complex environment, and in vitro folding will not necessarily reflect the native folding pathway. Additionally, a correlation between the rate of the in vitro folding and release from the cell has also been shown for B. subtilis α-amylase AmyE and levanel, although the binding affinity of Ca²⁺ to these proteins is very low [125].

The presence of the cell wall matrix, or some as yet unidentified wall component, impacts on the processing, release and, possibly, the post-translational folding of secreted proteins. Protoplasts prepared by lysozyme treatment of B. subtilis can efficiently translocate secretory proteins such as α-amylases, proteases and also human serum albumin [66,126–128]. However, less than 50% of the proteins expressed at high level were released from protoplasts [66,127,128]. In addition, some retardation in the processing of the signal peptide was observed, although all of the secreted proteins were processed [43,66]. Finally, the release of a protease was Mg₂⁺-dependent [126], unlike that of two different α-amylases [66,126].

4. Folding stress

The misfolding of proteins for any reason, or the synthesis of abnormal proteins, universally elicits cellular stress responses that ultimately lead to the refolding or degradation of the affected proteins. In bacteria there are distinct pathways to sense and respond to the presence of such proteins outside the cytoplasmic membrane, typically in the periplasm of Gram-negative bacteria or in the cell wall of Gram-positive bacteria. The degradation of such proteins due to this stress response is a major obstacle to the effective production of many heterologous secreted proteins in Gram-positive bacteria.

The best characterised bacterial extracytoplasmic stress response is that of E. coli (for a review, see Ref. [129]). There are two sensor–signal transduction pathways, based either on the regulation of sigma E activity or on the CpxAR two-component histidine kinase-response regulator. Both pathways sense misfolded or abnormal proteins in the periplasm, or the perturbed assembly of outer membrane proteins. In the sigma E pathway, the sensing mechanism involves interactions between the RseA anti-sigma factor in the cytoplasmic membrane and the periplasmic RseB protein. The CpxAR system involves an interaction between the CpxA histidine kinase in the cytoplasmic membrane and the periplasmic CpxP protein. The activation signals of both pathways are largely, but not exclusively, overlapping [129].
Both systems activate a regulon that includes a set of genes encoding periplasmic proteins with a function in protein folding, such as a disulfide bond isomerases, PPlases, or proteins associated with flagellar assembly. A prominent protein induced by both systems is the periplasmic DegP protein. DegP is a member of the ubiquitous family of HtrA serine proteases with dual functionality; their protease activity is required for the degradation of misfolded or aggregated proteins, while their chaperone function may allow the refolding of certain proteins at low temperature [130]. Both regulons are typically up-regulated by the synthesis of mutant periplasmic or outer membrane proteins, other perturbations of the assembly of the outer membrane and, characteristically, also by heat shock, ethanol starvation or oxidative stress. All these environmental insults may denature envelope proteins [129].

Sequence analyses of the genomes of *B. subtilis* [76] and a number of other Gram-positive bacteria have revealed proteins and sensor systems that are homologous to those induced by extracytoplasmic folding stress in *E. coli*. They include three HtrA homologues: HtrA (YkdA), HtrB (YvtA) and YycA (YyxK). These proteins are predicted to be associated with the cytoplasmic membrane with an N-terminal membrane spanning segment and a large extracytoplasmic domain containing the catalytic site and one PDZ domain [131,132]. One of the two-component systems identified in *B. subtilis* [133], consisting of the CssR (YvqA) response regulator and theCssS (YvqB) sensor kinase, shows significant sequence similarity (~50%) to the CpxAR system of *E. coli* [134]. It has been shown that CssRS of *B. subtilis* plays a role in the detection of extracytoplasmic misfolded proteins and in combating secretion or extracytoplasmic folding stress. In cssS- or cssR-null mutants, high-level secretion of α-amylase retarded growth in a manner that was dependent on the rate of α-amylase synthesis [134]. The effect was even more dramatic in a cssS prsA3 double mutant. The prsA3 mutation decreases the level of the PrsA extracytoplasmic folding factor about 10-fold. In the double mutant there was accumulation of enzymatically inactive and thus presumably harmful misfolded α-amylase. A direct link was also shown between the activity of the CssRS two-component system and the proteolytic degradation of abnormally folded extracellular proteins. Compared to wild-type PrsA, the PrsA3 mutant protein is present at reduced levels due to extracytoplasmic degradation. This degradation was significantly reduced in a cssS mutant [134].

In good agreement with the function of combating extracellular folding stress, the expression of the htrA and htrB genes of *B. subtilis* is controlled by CssRS. First, lacZ transcriptional fusion studies of the htrA or htrB genes show that high-level production of α-amylase (AmyQ, AmyL or a chimeric derivative of AmyL) drastically up-regulates both of these genes [49,132,134]. This response wasCssS-dependent [49,134], consistent with a critical role of CssRS in the induction of extracytoplasmic cleaning proteases following the sensing of extracytoplasmic folding stress. Induction of HtrA was also demonstrated through the proteomic analysis of secreted proteins [135].

As demonstrated using translational lacZ gene fusions, both htrA and htrB are strongly induced by heat shock, in a manner that is similar to the sigmaE or Cpx regulons of *E. coli* [131,132], and consistent with misfolding of secreted proteins at elevated temperature. However, high salt, ethanol or puromycin neither up-regulated htrA nor htrB, indicating the presence of a distinct and differently regulated heat shock regulon from those previously identified in *B. subtilis*. Heat shock induction of both genes is alsoCssRS-dependant, indicating that this system senses abnormal proteins regardless of the way they are generated [49].

The htrA, htrB and cssRS genes are also subject to an intriguing interactive regulation since the htrA and htrB genes are negatively autoregulated [131,132] at the transcriptional level. In the case of htrA, repression is also brought about by a mutant htrA gene encoding a proteolytically inactive HtrA protein [132]. Interestingly, there is also strong negative cross-regulation between htrA and htrB. Thus, heat induction of each gene is greatly enhanced in the mutant background of the other one [132]. There is a molecular basis for this cross-regulation in the form of high sequence homology between the promoters of these genes and the presence of similar negative regulatory elements in both promoters [132]. The autoregulation is also extended to the cssRS operon [49]. CssRS was autoregulated under conditions of secretion stress imposed on the cells either by overexpression of AmyQ, or by inactivating htrA or htrB. There is further cross-regulation between cssRS and htrB, which are expressed from juxtaposed promoters that seem to share regulatory regions [49].

The presence of paralogous HtrA-like proteins and the interactions between the corresponding genes seem to provide redundancy in the cells ability to combat extracytoplasmic protein folding stress. Mutations in any of the single genes encoding these components do not have significant effects on the cell phenotype. However, double mutations, such as cssS htrA, compromised growth [49], while htrA htrB double mutations exhibit decreased tolerance to thermal or oxidative stress [132]. In each case, the phenotype is presumably due to the cells having insufficient capacity to cope with misfolded or aggregated extracytoplasmic proteins. Autoregulation of signal transduction components is a common feature of bacteria to amplify a particular stress response, and cross-regulation may provide a means for its fine-tuning.

The presence of HtrA-like proteins has also been established in Gram-positive bacteria other than *B. subtilis* and a role in combating extracytoplasmic folding stress has also been proposed for them [136–139]. In this context, it has been suggested that HtrA-like proteins could also participate in proteolytic processes other than those related to the secretion stress response. Mutations in htrA homologues of *L. lactis* and *Streptococcus mutans* indicate a function in
the processing of extracytoplasmic proteins of structural importance for the cell surface [137], folding and proteolytic processing of exoproteins with prepeptides [138], and even the stability of secreted heterologous proteins [138]. Recent findings of Antelmann et al. [135] may suggest related functions for HtrA of \emph{B. subtilis}. It is noteworthy that, in addition to a membrane-associated form of HtrA, considerable amounts of proteolytically truncated HtrA were shown to be released into the growth medium. The extracellular amounts of HtrA were increased under conditions of htrA up-regulation. Under these conditions, a concomitantly increased appearance of the Yqxl protein of unknown function was observed in the growth medium. Remarkably, however, the level of extracellular Yqxl was determined post-transcriptionally in an HtrA expression-dependent manner. Moreover, the protease active site of HtrA turned out to be dispensable for this post-transcriptional Yqxl regulation. Based on these observations and the fact that HtrA of \emph{B. subtilis} shares a high degree of sequence similarity with HtrA of \emph{E. coli}, for which a chaperone-like activity has been demonstrated, it was proposed that both the proteolytic and chaperone-like activities are conserved in \emph{B. subtilis} HtrA. A chaperone-like activity of \emph{B. subtilis} HtrA could be involved in the extracellular appearance of Yqxl [140].

5. Conclusions

The fate of proteins emerging from the secretory translocase at the trans side of the cytoplasmic membrane is largely determined by their ability to fold rapidly in the micro-environment created at the membrane–wall interface. This environment is largely dominated by the immobilised high-density negative charge contributed primarily by the anionic polymers and the highly mobile counterbalancing metal cations. Also located in this environment are membrane- and wall-associated proteases that function to maintain the quality of the secreted proteins, and possibly to keep the translocase and cell wall free of misfolded or aggregated proteins. These proteases are induced in response to stresses that are likely to lead to protein misfolding. Proteins emerging from the translocase are therefore subjected to opposing activities that, ultimately, decide their fate. To some extent, this fate is different for native and heterologous proteins. Native proteins have evolved to coexist with the quality control machine that is designed to ensure that secretory proteins neither block the translocase nor grow sites within the cell wall. They fold very rapidly into their native structures that are resistant to the quality control proteases and which are compatible with the cell wall. Their rapid folding is aided by folding factors such as prepeptides, PrsA, disulfide bond forming enzymes and/or metal cations. These proteins are now free to interact with, or traverse, the cell wall. Reducing the folding kinetics of normally well secreted proteins leads to an increase in their susceptibility to proteolysis and a reduction in their yield. The fate of heterologous proteins is largely determined by their intrinsic folding kinetics in the environment at the membrane–wall interface, the ability of host folding factors to assist their folding and their compatibility with the cell wall. However, the increased understanding of the factors involved now holds out the prospect of engineering production strains for the high-level production of heterologous proteins.

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