Review

Bacillus protein secretion: an unfolding story

Colin R. Harwood[1] and Rocky Cranenburgh[2]

[1] Institute of Cell and Molecular Biosciences, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK
[2] Cobra Biomanufacturing, Stephenson Building, The Science Park, Keele ST5 5SP, UK

***Bacillus subtilis* and its close relatives are widely used for the production of enzymes for the detergent, food and beverage industries. These organisms not only produce an appropriate range of enzymes but also have the capacity to secrete them into the culture medium at high concentrations. Purification from the culture medium rather than from the cytoplasm considerably reduces downstream processing costs. In recent years, considerable effort has been aimed at developing *B. subtilis* as a host for the production of heterologous proteins. The folded state of the target protein at various stages of the secretion pathway has proved to be important.**

Secretion systems of *Bacillus*

Members of the genus *Bacillus* are prodigious producers of industrial enzymes, such as proteases and α-amylases, that are secreted across their single membrane system directly into the culture medium. Given the advantages of secretory systems in terms of production capacity ($\geq$20 g l^{-1}) and the structural authenticity and purity of the product, it is surprising that more use has not been made of this group of bacteria. However, most attempts to use *Bacillus* for the manufacture of heterologous proteins have met with limited success. The reasons for these failures are a combination of the properties of the secretion pathway, the *Bacillus* cell envelope, quality control proteases and the target proteins themselves.

Typically, bacteria secrete 5–10% of the proteins encoded on their chromosomes, primarily via the Sec-dependent secretion pathway [1]. In addition to macromolecular hydrolases that provide the cells with simple nutrients, these secreted proteins include enzymes involved in cell-wall synthesis and cell division. Consequently, the Sec-dependent secretion pathway is essential for viability. Gram-negative bacteria such as *Escherichia coli* have evolved specialized substrate-specific protein secretion pathways (e.g. types I, III and IV) for the translocation of proteins across their double-membrane system, but Gram-positive bacteria generally lack the specialized secretion pathways found in Gram-negative bacteria and instead rely on the ubiquitous Sec-dependent (Sec) and twin-arginine translocation (Tat) [2] pathways for the secretion of unfolded and folded proteins, respectively. This review focuses on the importance of the folded state of secretory proteins at the various stages in the Sec-dependent pathway [3,4].

Corresponding author: Harwood, C.R. (colin.harwood@ncl.ac.uk).

Signal peptides of *B. subtilis* and *E. coli*

An early event in the protein secretion pathway is the identification of substrates for targeting to the secretion apparatus. Secretory proteins are identified via a hydrophobic N-terminal extension, the signal peptide, that is removed during the later stages of the process [5,6]. The signal peptides of the Sec-dependent and Tat pathway substrates exhibit a similar structural organization: a positively charged N-terminus (N region) followed by a hydrophobic core (H region) and a short cleavage (C) region containing the target site for signal peptidase (Figure 1). Substrate proteins are cleaved by two classes of signal peptidase. Type I signal peptidases cleave the most abundant class of secretory substrates. In contrast to *E. coli* which has a single type I signal peptidase (Lep), *B. subtilis* has five enzymes (SipS, SipT, SipU, SipV and SipW). SipS and SipT are the main signal peptidases, and one or the other is required for viability, indicating overlapping substrate specificities [7]. The type I signal peptides (Figure 1) of Gram-positive bacteria are on average longer (~30 amino acid) and more hydrophobic than their Gram-negative counterparts (~25 amino acids) but have a similar consensus cleavage site (AXA↓) [8]. Attempts to maximize the secretion of heterologous proteins using optimal signal peptides have been unsuccessful [9], reflecting a lack of understanding of the complex interactions between the signal peptide and mature protein domains.

The absence of a membrane-enclosed periplasm (see below) means that Gram-positive bacteria have a higher proportion of lipoproteins than do their Gram-negative counterparts. The signal peptides of lipoproteins are cleaved by a type II signal peptidase (LspA) and share discrete characteristics that include shorter N and H regions and consensus cleavage sites (Lipobox) that are distinct from that of type I signal peptides: [LITAGMV]-[ASGTIMVF]-[AG]-↓C-[SGENTAQR] (Figure 1) [10,11]. The amino acid at the N-terminus of the mature lipoprotein is invariably a lipid-modified Cys residue that serves to tether the protein to the outer surface of the cytoplasmic membrane [12].

In the case of Tat substrates, the signal peptide includes the twin arginine motif (SRRxFLK) at the junction between the N and H regions (Figure 1). This motif has proved valuable for predicting Tat substrates in Gram-negative bacteria, but it is less reliable in Gram-positive bacteria [13]. Tat signal peptides are generally longer (~37 amino acids) and less hydrophobic than their counterparts in Sec substrates and often have basic residues in their C regions that might act as Sec avoidance signals [14].

0966-842X/$ – see front matter doi:10.1016/j.tim.2007.12.001 Available online 7 January 2008

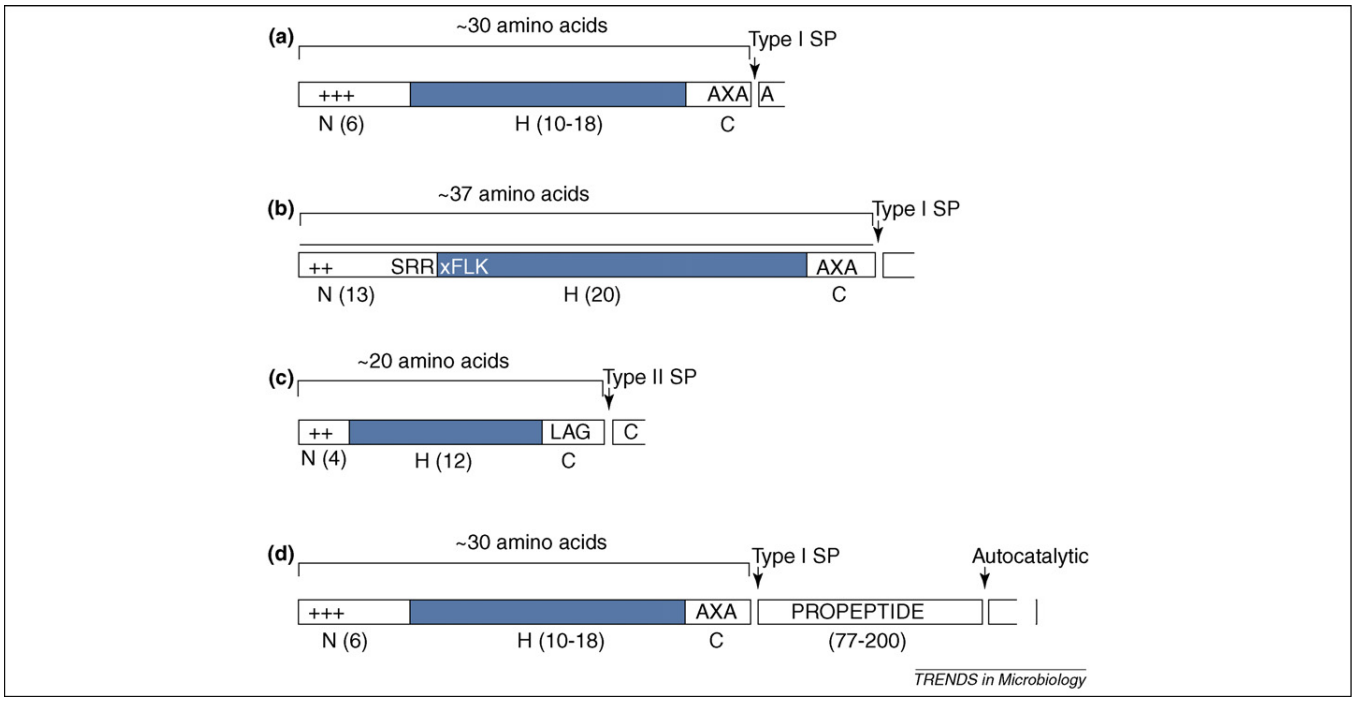

Figure 1. General features of the signal peptides and propeptides of *Bacillus* secretory proteins. The N-terminal (N), hydrophobic (H) and cleavage (C) regions are identified by contrasting shading and their lengths (amino acyl residues) are indicated in brackets. Cleavage sites are indicated by arrows. **(a)** Sec-dependent signal peptide cleaved by a type I signal peptidase (SP) at the AXA cleavage site. **(b)** Tat-dependent signal peptide with twin arginine motif (SRRxFLK), also cleaved by a type I SP. **(c)** Lipoprotein signal peptide cleaved by the type II SP. **(d)** The signal peptide and propeptide (prepropeptide) at the N-terminal end of a secretory protein requiring the propeptide for folding on the *trans* side of the cytoplasmic membrane.

Although signal peptides have been well characterized, surprisingly little is known about the molecular events associated with intracellular targeting and processing, particularly in Gram-positive bacteria. A key element of these intracellular events is the need to maintain Sec-dependent preproteins in a secretion-competent (i.e. unfolded) state, because proteins with significant amounts of tertiary structure are not able to pass through the translocase. By contrast, Tat pathway substrates can be translocated in a folded state.

Intracellular chaperoning

Sec-dependent secretory proteins are maintained in a secretion-competent state by intracellular chaperones. In *E. coli*, the signal recognition particle (SRP) pathway chaperoning or targeting system is complemented by the SecB and SecA chaperones that interact with a subset of SRP-independent secretory proteins [15]. *E. coli* SecB binds to the mature region of SecB-dependent presecretory proteins, and the resulting binary complex interacts with a specific site within the C-terminal region of SecA to form a tertiary complex that, in turn, interacts with the membrane-located secretory translocase. Conformational changes that result from the interaction of the tertiary complex with the secretory translocase lead to the release and recycling of SecB.

In common with other Gram-positive bacteria, *Bacillus* species lack a homologue of the secretion-specific chaperone SecB, and consequently the SRP pathway is the only recognized intracellular pathway for presecretory protein recognition, chaperoning and targeting [16]. The SRP is an RNA–protein complex that interacts with hydrophobic regions of signal peptides of nascent proteins, delivering them to a membrane-bound docking protein (FtsY), before passing the target protein to the secretory translocase. The detailed composition and mode of action of the SRP varies from one organism to another. In *Bacillus* species, the SRP consists of an RNA molecule (small cytoplasmic or scRNA) that provides the backbone for the attachment of two proteins: Ffh, so-called because of its similarity to the 54 kDa protein of eukaryotic SRPs, and HBsu, a histone-like DNA-binding protein (Figure 2). Ffh and FtsY are members of the SRP-GTPase protein family and are essential for viability.

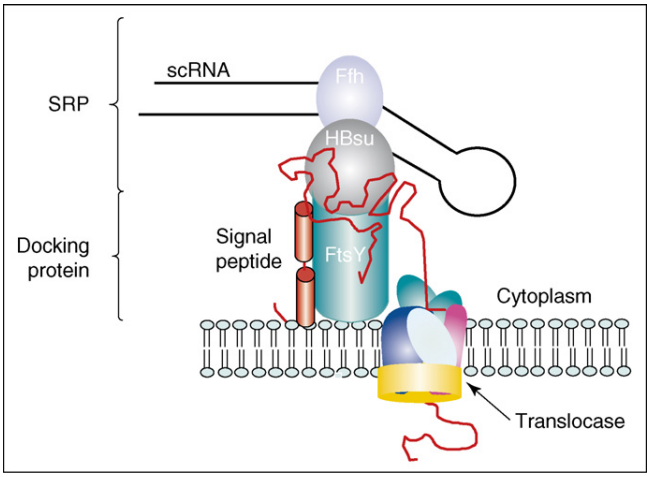

Figure 2. Schematic representation of the signal-recognition particle (SRP) of *B. subtilis*. Details of the interactions among the components of the SRP, the docking protein (FtsY) and the translocase are shown.

The more hydrophobic nature of the signal peptides of Gram-positive bacteria combined with the absence of a SecB homologue point to SRP playing an important role in the secretion of proteins from these bacteria [8]. Depletion of *B. subtilis* Ffh reduces Sec-dependent secretion [8], although experiments designed to elucidate the precise role and substrate specificity of the SRP pathway have provided ambiguous results.

Secretory preprotein (Sec-dependent) translocase

The Sec-dependent translocases of *E. coli* and *B. subtilis* show extensive similarities [17]. The SecY, SecE and SecG proteins form the core of a heterotrimeric integral membrane pore that interacts with SecA, the 'motor' component that drives translocation. SecA also has a role in chaperoning and targeting secretory substrates from their site of synthesis in the cytoplasm to the Sec translocase (Figure 3). SecA, SecY and SecE are essential for viability, whereas the absence of SecG leads to a cold-sensitive phenotype [18]. A second heterotrimeric complex, comprising SecDF–YajC in *E. coli,* appears to be important for SecA cycling, maintaining the forward momentum of preprotein substrates and their release on the *trans* side of the membrane [19]. In *B. subtilis*, SecDF is a natural fusion of the distinct SecD and SecF proteins of *E. coli*, whereas the *B. subtilis* homologue of YajC is YrbF [20]. A detailed description of the Sec-dependent translocase has been reviewed recently by Driessen and colleagues [17].

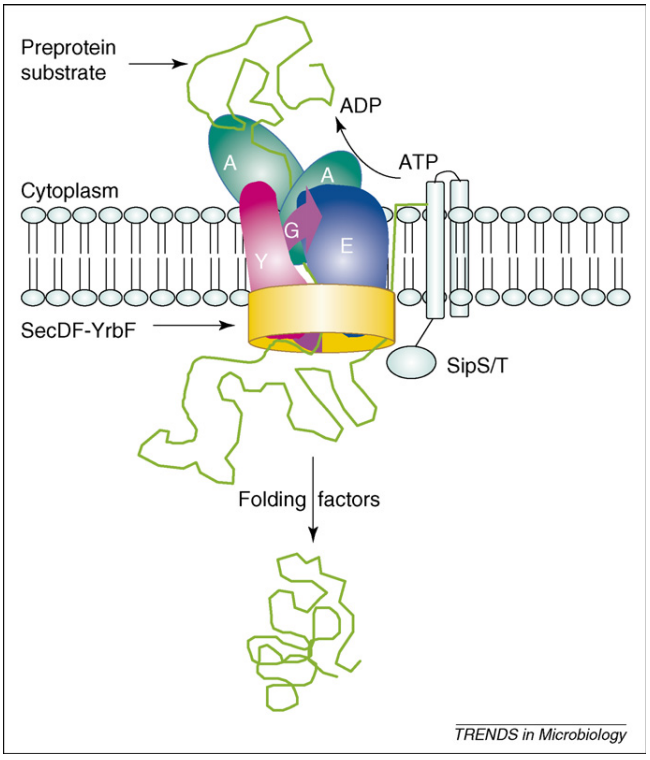

Figure 3. Schematic representation of the *B. subtilis* Sec-dependent secretory protein translocase. The preprotein substrate is transported through the translocase consisting of the SecA dimer, heterotrimeric pore (SecYEG), the heterotrimeric SecDF–YrbF complex and the main type I signal peptidases (SipS and SipT). After translocation, the preprotein is folded into its correct formation by propeptides, isomerases and divalent cations.

Many Gram-positive pathogens (e.g. *Bacillus anthracis, Staphylococcus aureus*, *Streptococcus gordonii* and *Streptococcus pneumoniae*) encode two SecA-like proteins, SecA and SecA2. The latter is thought to be required for the secretion of a subset of secretory proteins involved in pathogenesis [21,22].

Cell-wall structure and implications for secretion

Unlike their Gram-negative counterparts that have a double membrane system enclosing the cytoplasm, Gram-positive bacteria have a single membrane. Consequently, Gram-positive bacteria lack a membrane-enclosed periplasm that entraps a variety of enzymes and proteins at the cell's periphery (Box 1). Gram-positive bacteria compensate for the lack of a periplasm by tethering the homologues of many Gram-negative periplasmic proteins, such as rotamases and transporter substrate-binding proteins, to the outer surface of the cytoplasmic membrane by N-terminal lipo-modification. Other proteins (e.g. autolysins, wall- and surface-associated proteins) are targeted to the cell wall where they are tethered by ionic or, occasionally, covalent interactions. The combination of membrane and wall tethering provides a means of immobilizing specific proteins to a cell-associated but extracytoplasmic location that is functionally analogous to the periplasm of Gram-negative bacteria.

Proteins emerge from the Sec-dependent translocase on the *trans* side of the cytoplasmic membrane in a relatively unfolded state. Here, they encounter an environment that is dominated by the physicochemical properties of the Gram-positive cell wall. This environment includes a high density of immobilized negative charge, neutralized by mobile divalent metal cations (e.g. Ca^{2+}, Mg^{2+}, Fe^{2+}) – effectively a cation-exchange resin [23]. This is a challenging environment for the folding of secretory proteins, because they need to achieve their authentic structural

Box 1. Cell-wall structure

The cell walls of Gram-positive bacteria comprise a thick, highly cross-linked semi-porous murein sacculus that protects the underlying cytoplasmic membrane from an intracellular turgor of ~20 bar compared with 3–10 bar for *E. coli* [54,55]. Consequently, the membrane and cell wall are tightly apposed to each other. The *B. subtilis* cell wall is a multilayered structure that is both dynamic and flexible. In addition to its role in protecting the underlying protoplast and maintaining cell shape, the cell wall is involved in cell division, metal ion homeostasis and interactions between the cell and its environment [56].

The *Bacillus* cell wall is a copolymer of peptidoglycan and anionic polymers. Peptidoglycan, which typically represents ~40% of the wall by weight, is the structural component consisting of rigid glycan chains (repeating units of the disaccharide *N*-acetylglucosamine and *N*-acetylmuramic acid), cross-linked with flexible peptides via C3 of muramic acid. *B. subtilis* strain 168 contains two teichoic acids: the main polymer (~90% by weight) is poly(glycerolphosphate), which is essential for growth [57], whereas the minor polymer (~10%) of poly(glucosyl *N*-acetylgalactosamine 1-phosphate) is dispensable. Both anionic polymers are covalently attached to peptidoglycan at C6 of *N*-acetylmuramic acid via the same linkage unit [58]. Because teichoic-acid-containing cell walls can contain up to 30% of total cell phosphorus, teichoic acids are often extensively replaced with a non-phosphate-containing teichuronic acid during phosphate starvation [59]. The cell wall also contains significant amounts of lipoteichoic acid and protein.

configuration without forming aggregates with themselves, other proteins or the cell wall. The formation of protein aggregates at the inner surface of the cell wall is potentially catastrophic, because blockage of the cell-wall growth sites would, ultimately, lead to cell lysis.

Current evidence suggests that the rate of folding of proteins emerging from the secretory translocase is an important factor in minimizing aggregation and misfolding and maximizing yield. To this end, native secretory proteins fold rapidly, facilitated by a combination of intrinsic and extrinsic factors, such as chaperones and folding factors. Three types of folding factor have been identified in *B. subtilis* and related species: propeptides, isomerases and metal ions.

Propeptides

Several *Bacillus* secretory proteins, most notably proteases, have cleavable propeptides located in the primary translation product between their signal peptide and mature substrate protein (Figure 1). Propeptides vary from ~70 to 200 amino acid residues. The propeptide of subtilisin BPN' from *Bacillus amyloliquefaciens* is 77 residues (residues 31 to 107), whereas that of a low pI serine protease from *B. subtilis (natto)* is 164 residues (residues 31 to 194). Propeptides are not directly involved in protein translocation *per se*, but they are essential in the post-translocational folding required to achieve the active and stable form of their cognate secretory protein [24]. The propeptides of serine proteases serve two functions: they overcome large kinetic barriers in the productive folding pathway and are potent competitive inhibitors of the active enzyme [25].

During the secretion of a preproprotein, the signal peptide is cleaved in the usual manner during or immediately after translocation. In the absence of its propeptide, the folding of subtilisin is slow, presumably because of the low stability of intermediate folding states [26]. The role of the propeptide is, therefore, to accelerate folding by stabilizing an intermediate complex, thereby generating a nucleus for the completion of the folding pathway [26,27]. In the case of subtilisin, once the proprotein is folded, the propeptide remains closely apposed to the mature enzyme, temporally limiting the proteolytic activity of subtilisin. Full enzymatic activity is achieved only after the proteolytic self-cleavage and degradation of the propeptide [25]. Although propeptides are intramolecular chaperones, exogenously added purified propeptides are able to catalyse the folding of their cognate mature protein *in vitro*.

Propeptide-catalysed folding and subsequent removal of the propeptide are necessary for subtilisin to traverse the cell wall. Inactive precursor is retained in the cell, but is released to the growth medium by simultaneous production of active subtilisin [28]. An exception is a *Streptomyces* protease that is secreted in the culture medium in an inactive form with the propeptide attached [29]. The propeptide is eventually removed and the enzyme activated by a coexisting extracellular protease [30].

In a few secreted proteins (e.g. AmyE of *B. subtilis*) the signal peptide cleavage site is followed by a short stretch of amino acids that are not found in the mature protein. These 'propeptides' are dispensable for secretion, folding and stability and are removed nonspecifically in the culture supernatant by proteolytic nibbling [31].

Divalent cations as folding catalysts

The microenvironment into which secreted proteins are translocated is rich in metal cations, particularly Ca^{2+}, Fe^{3+} and Mg^{2+}. Many *Bacillus* secretory proteins are metalloproteins that require metal ions for folding, structural stability and activity. It has been demonstrated clearly that metal ions have a role in the post-translocational folding and secretion of a chimeric form of the *Bacillus licheniformis* α-amylase (AmyL) engineered to give a pI value of 10.0. An ~100-fold increase in the concentration of Ca^{2+} was required for the chimeric AmyL to achieve the same folding rate *in vitro* as that of the wild-type enzymes, and *in vivo* the chimeric AmyL was considerably more susceptible to extracytoplasmic quality control proteases (see below) [32,33].

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 D-alanylated. Inactivation of the *B. subtilis dlt* operon, responsible for the alanylation, increases the concentration of cations at the membrane–cell-wall interface, and this in turn increases the yield of many secretory proteins, including *B. amyloliquefaciens* and *B. licheniformis* amylases [34] and *B. anthracis* protective antigen [35].

Peptidyl prolyl *cis-trans* isomerases

In comparison with Gram-negative bacteria, relatively few proteins assist the post-translocational folding of Gram-positive secretory proteins. An exception is PrsA. *B. subtilis* PrsA is a lipoprotein of 270 amino acid residues [36] and is essential for growth. Depletion ultimately causes gross morphological alterations and cell death. Depletion of PrsA also causes a marked reduction in the production of the *B. amyloliquefaciens* α-amylase AmyQ by *B. subtilis,* whereas overproduction leads to a dramatic increase in the production of this enzyme. However, most secretory proteins are PrsA independent [37]. Although no PrsA-dependent substrates have been unambiguously identified in *B. subtilis,* it is assumed that one such protein involved in cell-wall synthesis is responsible for the essential phenotype [38]. However, the production of *B. licheniformis* and *B. amyloliquefaciens* α-amylases, AmyL and AmyQ, and the *B. anthracis* protective antigen, PagA, by *B. subtilis* have been shown to be PrsA dependent. [38]

Three PrsA-like proteins (PrsAA, PrsAB and PrsAC) have been identified in *B. anthracis* [39]. When expressed in *B. subtilis,* all three proteins complemented the function of *B. subtilis* PrsA. The proteins appear to have distinct but overlapping substrate specificities. The *B. subtilis* genome also encodes a paralogue of PrsA, namely YacD, which is incapable of complementing PrsA with respect to either viability or secretion.

PrsA does not influence the rate of translocation, and strains depleted of PrsA do not accumulate non-processed precursors. Instead, strains with reduced concentrations of PrsA showed marked increases in the post-translocation degradation of PrsA-dependent proteins. These data support the view that PrsA functions as an extracellular, but

cell-associated, folding chaperone or foldase. The molecular mechanism by which PrsA facilitates the folding of target proteins remains to be elucidated. However, PrsA shows sequence similarity to peptidyl-prolyl *cis–trans* isomerases (PPIases) of the parvulin family [37,40]. PPIases increase the rate of folding of proteins with *cis*-prolyl residues, and this is consistent with the function of PrsA in enhancing the folding of secreted proteins and reducing their susceptibility to proteolysis.

Disulphide isomerases

The secretory proteins of *B. subtilis* and other Gram-positive bacteria generally lack disulfide bonds, and consequently relatively little is known about disulfide-bond formation. The two native peptides or proteins that are known to form disulphide bonds, the bioactive sublancin peptide and pilin-like ComGC protein required for competence development, are not secreted via the Sec pathway. Three *B. subtilis* thiol-disulfide oxidoreductase enzymes involved in disulfide-bond formation are likely to be membrane proteins, and a fourth enzyme, BdbD, has a predicted signal peptide and is likely to be secreted. These enzymes are relevant to the commercial exploitation of *Bacillus* species because the absence of BdbB or BdbC results in a reduction in the efficiency of disulfide formation in the heterologous *E. coli* alkaline phosphatase, PhoA [41].

Extracytoplasmic quality control proteases

The failure of a secretory protein to be released from the translocase or their illegitimate interaction with cell wall growth sites are both potentially fatal events. As a result, *B. subtilis* encodes 'quality control' proteases that monitor proteins at the membrane and wall interface, degrading those that are misfolded. The main proteins involved are the serine proteases WprA, HtrA and HtrB [42,43]. Signal transduction pathways are responsible for sensing and inducing these enzymes in response to secretion and physiological (e.g. heat) stresses that are likely to influence the structure of secretory proteins. The presence of these quality control proteases appears to be a major barrier to the production of many heterologous proteins secreted by Gram-positive bacteria.

Wall-associated protein A (WprA) is the processed product of the *wprA* gene [43]. The primary translation product is 96 kDa, but is proteolytically processed after translocation. Two processed products are detected in cell-wall extracts: cell wall binding protein (CWBP) 23 is derived from the N-terminal portion of the primary polypeptide product, and CWBP52 is derived from the C terminus. The ~21 kDa linker region that connects CWBP23 and CWBP52 has not been detected in the cell wall and is presumably rapidly degraded. CWBP52 is a serine protease involved in the degradation of non-native secretory proteins, whereas CWBP23 is likely to be a propeptide involved in both the folding of CWBP52 and the control of its activity. *wprA*-null mutants have no obvious growth phenotype but show enhanced production of native wall proteins and certain heterologous proteins [43]. The involvement of WprA in the thermal inactivation of a temperature-sensitive derivative signal peptidase S indicates that WprA is active at the interface between the membrane and the cell wall [44]. *wprA* is regulated by YvrG-YvrHb, a two-component signal transduction pathway that also controls the expression of genes encoding the major cell-wall autolysins [45]. However, the signal responsible for the induction of this system is not well understood, although there is evidence that *wprA* is induced in response to secretion stress (C. Harwood and R. Cranenburgh, unpublished).

Homologues of the *E. coli* HtrA and HtrB proteases responsible for the degradation of misfolded periplasm proteins have been identified in *B. subtilis* [42]. It is predicted that the *B. subtilis* proteins are membrane anchored and have large extracytoplasmic serine protease domains.

The genes encoding these proteins are induced in response to secretion stress (e.g. overproduction of AmyQ) and heat shock via the CssR–CssS two-component signal transduction pathway (Figure 4) [46]. CssRS, therefore, detects misfolded proteins at the interface between the membrane and wall irrespective of how they are generated. The resulting induction of HtrA and HtrB reduces the potential for misfolded proteins to block the translocase and/or cell wall growth sites.

Although *cssS*- or *cssR*-null mutants are viable, high levels of secretion of AmyQ result in a significant reduction in growth rate [47]. Similarly, mutations in either of the genes encoding HtrA and HtrB do not have a noticeable effect on the growth or secretory protein yield. However, the absence of HtrA leads to the increased synthesis of HtrB and vice versa [48]. The *htrA- htrB*-null double mutant exhibits a marked sensitivity to thermal or oxidative stress and shows a noticeably reduced growth rate and yield of secretory proteins [48]. There is evidence that *B. subtilis* HtrA has chaperone-like activity that might assist misfolded proteins to recover their configuration, while targeting unsuccessful protein for degradation [49].

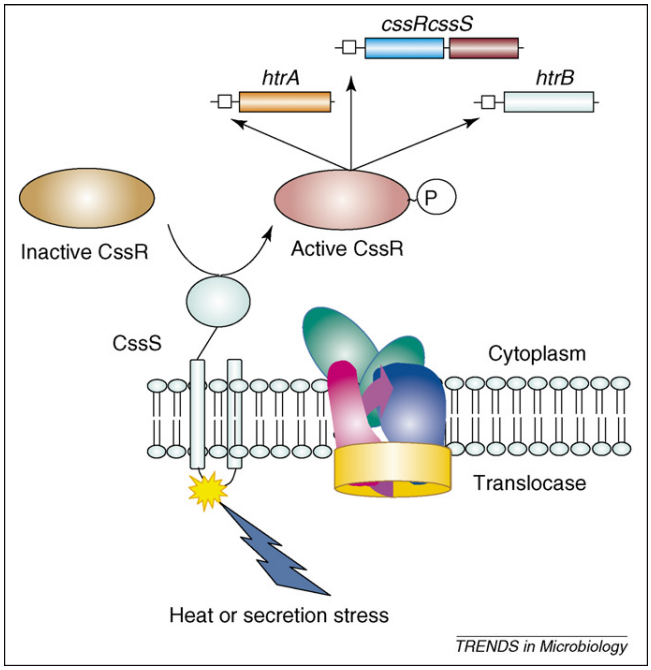

Figure 4. Induction of the CssRS two-component signal transduction pathway. CssRS senses secretion stress or heat. Activated CssR induces the expression of *htrA* and *htrB* as well as upregulating expression of the *cssRS* operon.

Concluding remarks and future directions

B. subtilis and its relatives are widely used for the production of industrial enzymes. The absence of an outer membrane combined with an efficient Sec-dependent secretion pathway means that proteins can be secreted directly into the culture medium at high concentrations. Disappointingly, from a commercial perspective, these bacteria are less successful at secreting heterologous proteins, and yields often drop to milligram or even microgram quantities per litre of culture. Understanding the reasons for the bottleneck in heterologous protein secretion has, coincidentally, revealed a great deal of molecular information about the *Bacillus* Sec-dependent secretion pathway.

The environment into which secretory proteins emerge after translocation across the cytoplasmic membrane is unique to Gram-positive bacteria. A key observation is that proteins must fold rapidly if they are to avoid blocking the translocase itself or the cell-wall growth sites: slowly folding proteins expose protease-sensitive sites that are not exposed in the fully folded protein. This is best illustrated by the kinetics of secretion of *B. licheniformis* amylase AmyL [50]. Pulse-chase experiments show that during secretion from *B. licheniformis* almost 100% of the synthesized protein is recovered from the culture medium. By contrast, when transferred to *B. subtilis,* ~75% of the initially formed protein is degraded, and only 25% is recovered from the growth medium. Degradation takes place after the removal of the signal peptide but before the protein is released into the culture medium, and WprA is responsible for a significant portion of this degradation [43]. This degradation is even more dramatic when a folding mutant of AmyL is used. Both AmyL and its folding mutant are, nevertheless, completely stable in the presence of proteases in the culture medium. These data imply that native secretory proteins have co-evolved with their natural host to avoid both the quality control proteases and, in the case of *B. subtilis,* the seven proteases secreted into the culture medium to provide nutrients from the degradation of proteins and peptides in the environment (i.e. feeding proteases). The challenge is to tailor the *B. subtilis* secretion pathway for the secretion of high concentrations of heterologous protein by avoiding these cellular 'booby traps'. One problem is the formation of secretion-incompetent folded intermediates of secretory proteins in the cytoplasm. Wu and colleagues have attempted to overcome this problem by generating strains in which the synthesis of intracellular chaperones such as GroES and GroEL or DnaK, DnaJ and GrpE is upregulated [51]. This was achieved by inactivating the gene encoding HrcA, the negative regulator of the *groE* and *dnaK* operons. This increased the yields of the single-chain antibody (SCA) fragment based on a fibrin-specific monoclonal antibody MH-1 [51] and an anti-digoxin-SCA [52] from *B. subtilis* and reduced inclusion body formation of anti-digoxin-SCA [52]. Increasing the production of PrsA can improve the recovery of the limited number of proteins that are substrates for this chaperone, presumably by reducing their susceptibility to proteolysis. These proteins include α-amylases [53], *B. anthracis* protective antigen [39] and MH-1-SCA [51].

Many heterologous proteins are secreted efficiently from *B. subtilis* but are susceptible to the numerous extracellular quality control (WprA, HtrA and HtrB) and feeding (NprB, AprE, Epr, Bpr, NprE, Mpr and VprA) proteases encoded by this bacterium. An obvious approach is to generate strains in which the genes encoding proteases have been deleted, and Wong and colleagues developed a series of strains in which the feeding proteases and WprA were deleted [51]. Such strains have been helpful in improving the productivity of *B. subtilis* for the production of single-chain antibodies against some antigens (e.g. anti-digoxin-SCA) but not others (e.g. fibrin-specific monoclonal antibody, MH-1-SCA), reflecting the fact that a relatively subtle difference in folding and structure can markedly affect the secretion characteristics and yield of individual proteins. More recently, a fully antibiotic-sensitive strain was generated in which the seven extracellular and three quality control proteases were precisely and completely excised from the chromosome (R. Cranenburgh and C. Harwood, unpublished). This strain provides a useful basis for the exploitation of synthetic biology approaches for the development of commercial strains optimized for the production of native and heterologous proteins. Ultimately, this will involve the deletion of significant portions of the chromosome-encoding genes not required for the growth of *B. subtilis* in complex media (e.g. prophage-like, sporulation and secondary metabolite genes) and enhancing the production of proteins involved in all stages of the protein secretion pathways (e.g. chaperones and translocase components).

Acknowledgements

The authors acknowledge funding from the UK BBSRC/DTI Link scheme (BBS/B/13799) and from the European Union (Bacell Health: LSH-2002–1.1.0–1).

References

1 Holland, I.B. (2004) Translocation of bacterial proteins – an overview. *Biochim. Biophys. Acta* 1694, 5–16
2 Robinson, C. and Bolhuis, A. (2004) Tat-dependent protein targeting in prokaryotes and chloroplasts. *Biochim. Biophys. Acta* 1694, 135–147
3 Sarvas, M. *et al.* (2004) Post-translocational folding of secretory proteins in Gram-positive bacteria. *Biochim. Biophys. Acta* 1694, 311–327
4 Tjalsma, H. *et al.* (2004) Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* 68, 207–233
5 Bendtsen, J.D. *et al.* (2005) Prediction of twin-arginine signal peptides. *BMC Bioinformatics* 6, 167
6 Nielsen, H. *et al.* (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10, 1–6
7 Tjalsma, H. *et al.* (1999) The plasmid-encoded signal peptidase SipP can functionally replace the major signal peptidases SipS and SipT of *Bacillus subtilis*. *J. Bacteriol.* 181, 2448–2454
8 Zanen, G. *et al.* (2005) Signal peptide hydrophobicity is critical for early stages in protein export by *Bacillus subtilis*. *FEBS J.* 272, 4617–4630
9 Brockmeier, U. *et al.* (2006) Systematic screening of all signal peptides from *Bacillus subtilis*: A powerful strategy in optimizing heterologous protein secretion in Gram-positive bacteria. *J. Mol. Biol.* 362, 393–402
10 Sutcliffe, I.C. and Harrington, D.J. (2002) Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. *Microbiology* 148, 2065–2077
11 Tjalsma, H. and van Dijl, J.M. (2005) Proteomics-based consensus prediction of protein retention in a bacterial membrane. *Proteomics* 5, 4472–4482
12 Juncker, A.S. *et al.* (2003) Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci.* 12, 1652–1662

13 Jongbloed, J.D.H. *et al.* (2002) Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*. *J. Biol. Chem.* 277, 44068–44078
14 Cristóbal, S. *et al.* (1999) Competition between Sec- and TAT-dependent protein translocation in *Escherichia coli*. *EMBO J.* 18, 2982–2990
15 Luirink, J. and Sinning, I. (2004) SRP-mediated protein targeting: structure and function revisited. *Biochim. Biophys. Acta* 1694, 17–35
16 Zanen, G. *et al.* (2006) Proteomic dissection of potential signal recognition particle dependence in protein secretion by *Bacillus subtilis*. *Proteomics* 6, 3636–3648
17 de Keyzer, J. *et al.* (2003) The bacterial translocase: a dynamic protein channel complex. *Cell. Mol. Life Sci.* 60, 2034–2052
18 van Wely, K.H.M. *et al.* (1999) Functional identification of the product of the *Bacillus subtilis yvaL* gene as a SecG homologue. *J. Bacteriol.* 181, 1786–1792
19 Nouwen, N. *et al.* (2005) The large first periplasmic loop of SecD and SecF plays an important role in SecDF functioning. *J. Bacteriol.* 187, 5857–5860
20 Bolhuis, A. *et al.* (1998) SecDF of *Bacillus subtilis*, a molecular siamese twin required for the efficient secretion of proteins. *J. Biol. Chem.* 273, 21217–21224
21 Bensing, B.A. *et al.* (2005) Determinants of the streptococcal surface glycoprotein GspB that facilitate export by the accessory Sec system. *Mol. Microbiol.* 58, 1468–1481
22 Kurtz, S. *et al.* (2006) The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. *Infect. Immun.* 74, 6855–6864
23 Beveridge, T.J. and Murray, R.G. (1980) Sites of metal deposition in the cell wall of *Bacillus subtilis*. *J. Bacteriol.* 141, 876–887
24 Yabuta, Y. *et al.* (2002) Folding pathway mediated by an intramolecular chaperone: dissecting conformational changes coincident with autoprocessing and the role of Ca^{2+} in Subtilisin maturation. *J. Biochem. (Tokyo)* 131, 31–37
25 Yabuta, Y. *et al.* (2001) Folding pathway mediated by an intramolecular chaperone. Propeptide release modulates activation precision of pro-subtilisin. *J. Biol. Chem.* 276, 44427–44434
26 Wang, L. *et al.* (1998) Engineering the independent folding of the subtilisin BPN' pro-domain: correlation of pro-domain stability with the rate of subtilisin folding. *Biochemistry* 37, 3165–3171
27 Gallagher, T. *et al.* (1995) The prosegment-subtilisin BPN' complex: crystal structure of a specific 'foldase'. *Structure* 3, 907–914
28 Power, S.D. *et al.* (1986) Secretion and autoproteolytic maturation of subtilisin. *Proc. Natl Acad. Sci. USA* 83, 3096–3100
29 Nagamine-Natsuka, Y. *et al.* (1995) Molecular cloning, nucleotide sequence, and expression of the gene encoding a trypsin-like protease from *Streptomyces erythraeus*. *J. Biochem. (Tokyo)* 118, 338–346
30 Chang, S.C. *et al.* (1997) Roles of the signal peptide and mature domains in the secretion and maturation of the neutral metalloprotease from *Streptomyces cacaoi*. *Biochem. J.* 321, 29–37
31 Takase, K. *et al.* (1988) NH_2-terminal processing of *Bacillus subtilis* alpha-amylase. *J. Biol. Chem.* 263, 11548–11553
32 Stephenson, K. *et al.* (1998) The influence of protein folding on the secretion of alpha-amylases from *Bacillus subtilis*. *FEBS Lett.* 430, 385–389
33 Stephenson, K. *et al.* (2000) The influence of secretory protein charge on late stages of secretion from the Gram-positive bacterium *Bacillus subtilis*. *Biochem. J.* 350, 31–39
34 Hyyryläinen, H-L. *et al.* (2000) D-alanine substitution of teichoic acids as a modulator of protein folding and stability in the cytoplasmic membrane–cell wall interface in *Bacillus subtilis*. *J. Biol. Chem.* 275, 26696–26703
35 Thwaite, J.E. *et al.* (2002) Optimization of the cell wall microenvironment allows increased production of recombinant *Bacillus anthracis* Protective Antigen (rPA) from *Bacillus subtilis*. *Appl. Environ. Microbiol.* 68, 227–234
36 Kontinen, V.P. *et al.* (1991) A gene (*prsA*) of *Bacillus subtilis* involved in a novel, late stage of protein export. *Mol. Microbiol.* 5, 1273–1283
37 Vitikainen, M. *et al.* (2004) Structure–function analysis of PrsA reveals 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
38 Vitikainen, M. *et al.* (2001) Quantitation of the capacity of the secretion apparatus and requirement for PrsA in growth and secretion of alpha-amylase in *Bacillus subtilis*. *J. Bacteriol.* 183, 1881–1890
39 Williams, R.C. *et al.* (2003) Production of *Bacillus anthracis* protective antigen is dependent on the extracellular chaperone. *PrsA. J. Biol. Chem.* 278, 18056–18062
40 Tossavainen, H. *et al.* (2006) NMR solution structure and characterization of substrate binding site of the PPIase domain of PrsA protein from *Bacillus subtilis*. *FEBS Lett.* 580, 1822–1826
41 Meima, R. *et al.* (2002) The *bdbDC* operon of *Bacillus subtilis* encodes thiol-disulfide oxidoreductases required for competence development. *J. Biol. Chem.* 277, 6994–7001
42 Darmon, E. *et al.* (2002) A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. *J. Bacteriol.* 184, 5661–5671
43 Stephenson, K. and Harwood, C.R. (1998) Influence of a cell-wall-associated protease on production of alpha-amylase by *Bacillus subtilis*. *Appl. Environ. Microbiol.* 64, 2875–2881
44 Bolhuis, A. *et al.* (1999) Different mechanisms for thermal inactivation of *Bacillus subtilis* signal peptidase mutants. *J. Biol. Chem.* 274, 15865–15868
45 Serizawa, M. *et al.* (2005) Functional analysis of the YvrGHb two-component system of *Bacillus subtilis*: Identification of the regulated genes by DNA microarray and northern blot analyses. *Biosci. Biotechnol. Biochem.* 69, 2155–2169
46 Westers, H. *et al.* (2006) The CssRS two-component regulatory system controls a general secretion stress response in *Bacillus subtilis*. *FEBS J.* 273, 3816–3827
47 Hyyrylainen, H-L. *et al.* (2001) A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. *Mol. Microbiol.* 41, 1159–1172
48 Noone, D. *et al.* (2001) YkdA and YvtA, HtrA-Like serine proteases in *Bacillus subtilis*, engage in negative autoregulation and reciprocal cross-regulation of *ykdA* and *yvtA* gene expression. *J. Bacteriol.* 183, 654–663
49 Antelmann, H. *et al.* (2003) The extracellular proteome of *Bacillus subtilis* under secretion stress conditions. *Mol. Microbiol.* 49, 143–156
50 Jensen, C.L. *et al.* (2000) Cell-associated degradation affects the yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system. *Microbiology* 146, 2583–2594
51 Wu, S-C. *et al.* (2002) Functional production and characterization of a fibrin-specific single-chain antibody Fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl. Environ. Microbiol.* 68, 3261–3269
52 Wu, S-C. *et al.* (1998) Enhanced secretory production of a single-chain antibody fragment from *Bacillus subtilis* by coproduction of molecular chaperones. *J. Bacteriol.* 180, 2830–2835
53 Kontinen, V.P. and Sarvas, M. (1993) The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level secretion. *Mol. Microbiol.* 8, 727–737
54 Csonka, L.N. and Epstein, W. (1996) Osmoregulation. In *Escherichia coli and Salmonella Cellular and Molecular Biology* (Neidhardt, F.C., ed.), pp. 1210–1223, ASM Press
55 Whatmore, A.M. and Reed, R.H. (1990) Determination of turgor pressure in *Bacillus subtilis*: a possible role for K^+ in turgor regulation. *J. Gen. Microbiol.* 136, 2521–2526
56 Foster, S.J. and Popham, D.L. (2001) Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers and capsules. In *Bacillus subtilis and its Closest Relatives: from Genes to Cells* (Sonenshein, A.L. *et al.*, eds), pp. 21–41, ASM
57 Bhavsar, A.P. *et al.* (2004) Teichoic acid is an essential polymer in *Bacillus subtilis* that is functionally distinct from teichuronic acid. *J. Bacteriol.* 186, 7865–7873
58 Freymond, P-P. *et al.* (2006) Poly(glucosyl-*N*-acetylgalactosamine 1-phosphate), a wall teichoic acid of *Bacillus subtilis* 168: its biosynthetic pathway and mode of attachment to peptidoglycan. *Microbiology* 152, 1709–1718
59 Qi, Y. and Hulett, F.M. (1998) Role of PhoP∼P in transcriptional regulation of genes involved in cell wall anionic polymer biosynthesis in *Bacillus subtilis*. *J. Bacteriol.* 180, 4007–4010