**Bacillus** protein secretion: an unfolding story

Colin R. Harwood¹ and Rocky Cranenburgh²

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 (≥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].

**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 (LepA) 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]-[SGENATAQR] (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].
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 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.

**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 (SRxFLK), also cleaved by a type I SP. (c) Lipoprotein signal peptide cleaved by a 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.

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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 preserreotary 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 a 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.

**Secretony 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 prepore 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](image-url)

**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(glycerol N-acetylglucosamine 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 overcame 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 preprotein 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 Ca²⁺, Fe³⁺ and Mg²⁺. 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 Ca²⁺ 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 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 isomerase (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, so are not relevant in the context of the Sec pathway. Three B. subtilis thiol-disulphide 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 periplasmic 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.](image)

CssRS senses secretion stress or heat. Activated CsrS 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 feeding proteases and WprA 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. prophase-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).

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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].
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