# Improving protein secretion by engineering components of the bacterial translocation machinery

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The increased insight into the mechanism of bacterial protein translocation has resulted in new concepts for the production of heterologous proteins. The periplasm of Gram-negative bacteria is revealed to have a role as a 'protein construction compartment', which can be used to fold complex proteins. Passage across the outer membrane, however, remains a challenge due to the high selectivity of the outer membrane translocase. In Gram-positive bacteria, slow folding at the membrane–cell-wall interface can make heterologous proteins vulnerable to degradation by wall-associated proteases. The recent identification of thiol-disulfide oxidoreductases in *Bacillus subtilis* might open the possibility of secreting proteins containing multiple disulfide bonds from this host.

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**Abbreviations**

| | |
|---|---|
| **Dsb** | thiol-disulfide oxido-reductase |
| **GSP** | general secretion pathway |
| **Lif** | lipase-specific foldase |
| **MTB** | main terminal branch |
| **PPI** | peptidyl-prolyl-*cis-trans*-isomerase |
| **scFv** | single chain variable region fragment |

## Introduction

Microbial host strains are attractive for the large-scale manufacturing of commercially relevant proteins due to their fast growth rate and their high protein synthesis capacity. Enhanced levels of gene expression, however, often result in the intracellular accumulation of inactive protein aggregates, also known as inclusion bodies. Active protein can be recovered from these aggregates only by tedious procedures with usually low yields.

Despite the drawback of inclusion body formation the majority of microbial-made biopharmaceuticals, including insulin [P1,P2] and human growth hormone [1], is still produced with the Gram-negative bacterium *Escherichia coli* as a host. Export of overexpressed heterologous proteins from the cytoplasm has been proposed as a solution for the prevention of inclusion body formation and the production of functional proteins in an easily recoverable form. With the identification of some periplasmic chaperone and foldase functions in *E. coli* the concept of using the periplasm as a 'construction compartment' in which chaperones aid the folding and functional assembly of proteins has come within reach. This will be further discussed in this review. The ultimate goal from the viewpoint of industrial-scale recovery —accumulation of proteins at gram per litre scale in the extracellular medium — requires, however, the passage of protein across two membranes. Recently, some non-pathogenic species as *Pseudomonas alcaligenes* have been described that have the capacity to secrete commercially important enzymes, such as lipases, proteases, cellulases and phospholipases, in significant amounts into the extracellular medium [2]. The outer membrane secretion machinery is crucial for the export of proteins from the periplasm. At high expression levels, the outer membrane can become a barrier, as exemplified by the formation of inactive periplasmic inclusion bodies upon overexpression of *Pseudomonas glumae* lipase.

*Bacillus subtilis* and related bacilli are attractive hosts for heterologous protein production as these Gram-positive eubacteria lack an outer membrane and, therefore, can secrete homologous and (some) heterologous proteins directly, at gram per litre concentrations, into the growth medium (see [3,4]). Moreover, *B. subtilis* is non-pathogenic, well-known with respect to fermentation technology [4] and now has a 'transparent' (i.e. sequenced) genome [5]. The *Bacillus* secretion system, however, has been shown to be recalcitrant with respect to heterologous proteins. Recently, the understanding of the molecular mechanism of secretion has been greatly improved and concepts for engineering the machinery towards improved secretion have been developed within the framework of several European Union projects. In this review, we will discuss the added value of having both a Gram-negative and a Gram-positive secretion host.

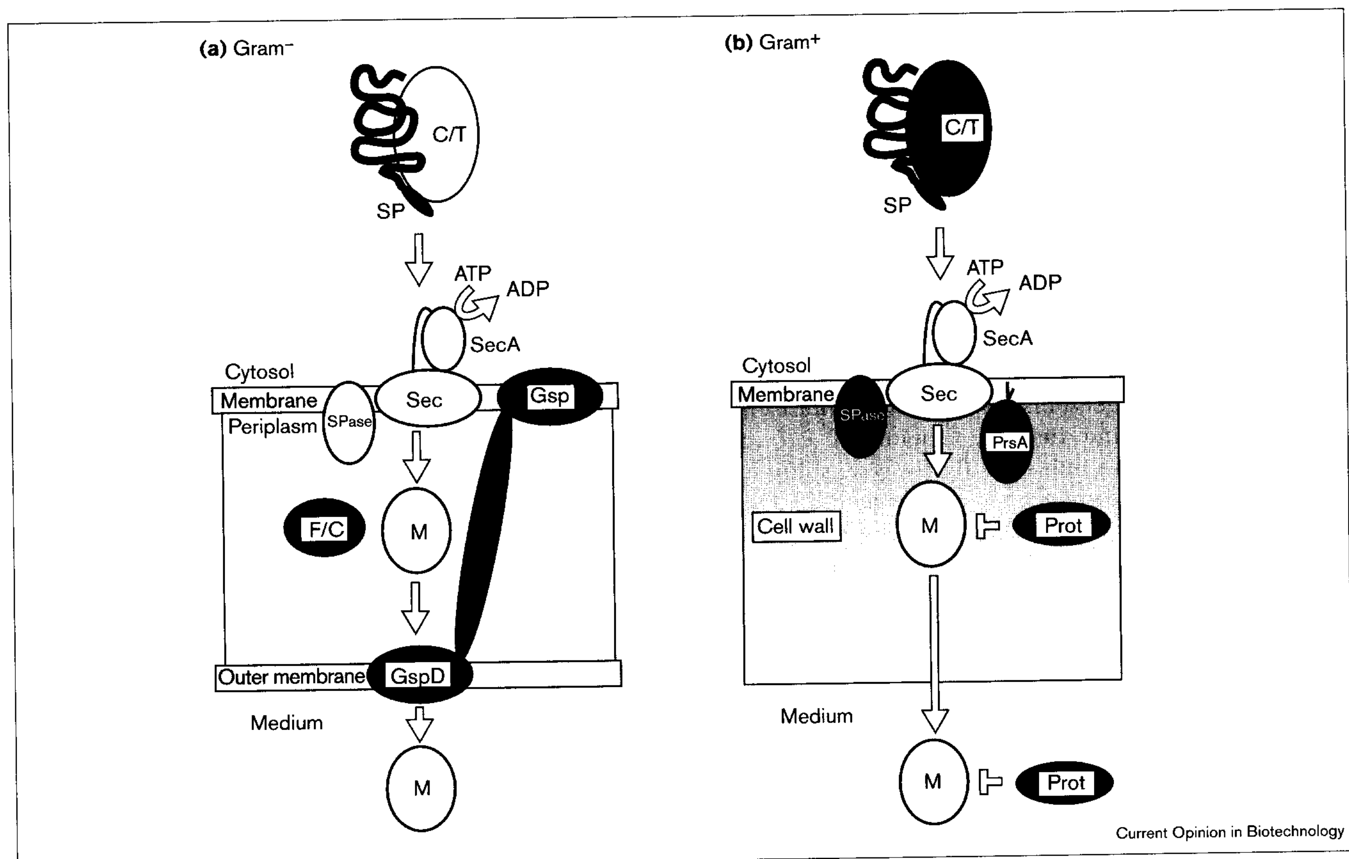
## Towards a general Gram-negative secretion host?

Secretion of proteins into the extracellular medium by Gram-negative bacteria requires the passage of both inner and outer membranes. Four different routes for this passage have been described, referred to as the type I, II, III and IV secretion pathways (see [6]). We will focus on the type II secretion pathway of *Pseudomonas* species, as these are efficient secretion hosts. Proteins secreted via the type II secretion pathway, also referred to as the main terminal branch (MTB) of the general secretion pathway (GSP), pass the cell envelope in two separate steps. First, they are translocated across the inner membrane into the periplasm, a process mediated by the Sec machinery. Subsequently, the periplasmic intermediates are translocated across the outer membrane as fully folded proteins (Figure 1).

### Cytosolic chaperones

Although the role of cytosolic SecB in the targeting of several *E. coli* proteins has been confirmed, little new information on the role of SecB in the secretion of

**Figure 1**




The general secretion pathway in **(a)** Gram-negative and **(b)** Gram-positive eubacteria. Secretory proteins are synthesized as precursors with an amino-terminal signal peptide (SP). Cytoplasmic chaperones (C) and targeting factors (T) keep the precursors in a translocation-competent conformation and facilitate their targeting to the Sec translocon in the membrane. Known components of the Sec translocon are SecA, SecY, SecE, SecG, SecDF and YajC. The SecYEGDFYajC complex forms the translocation channel (Sec). SecA acts as a force-generator (motor) for protein translocation through cycles of preprotein binding, membrane insertion, preprotein release and deinsertion from the membrane. SecA cycling is regulated by ATP-binding and hydrolysis (see [35]). During or shortly after translocation, precursors are processed by type I signal peptidases (SPase). Folding of the mature proteins (M) into their native conformation depends on the activity of specific and non-specific foldases and chaperones (F/C), such as the Lif, Dsb and Skp proteins from Gram-negative bacteria, or the lipoprotein PrsA of *B. subtilis*. Secretion of proteins from the periplasm of Gram-negative bacteria into the growth medium involves the main terminal branch of the general secretion pathway, consisting of 12–15 inner and outer membrane-associated components (Gsp; see [6]). A homomultimeric ring-shaped complex of $XcpQ_{GspD}$ (GspD) facilitates protein passage across the outer membrane. In Gram-positive bacteria, mature proteins are directly released into the growth medium upon translocation across the membrane and passage through the cell wall. Translocated and/or secreted mature proteins are challenged by proteases (Prot). Cellular components that have been engineered successfully for improved production of proteins are depicted in dark grey shading.

heterologous proteins has been gathered. Deployment of efficient signal peptides, such as those from alkaline phosphatase (PhoA) and pectate lyase (PelB), fused to a heterologous protein is a widely used pragmatic approach to target proteins into the Sec machinery. Whether the aggregation of secretory proteins in the cytoplasm can also be caused by a limited transport capacity of the preprotein translocase in the membrane is presently not known.

### Periplasmic chaperones

Several non-specific chaperones function in the periplasm of *E. coli*. The peptidyl-prolyl-*cis-trans*-isomerases (PPIs), catalyze the *cis-trans* isomerisation of X–proline peptide bonds (where X is any amino acid) [7,8]. This has been shown to be essential for the functional assembly of several *E. coli* outer membrane proteins. Upon high-level production of functional single chain variable region fragments (scFvs) in the periplasm of *E. coli*, isomerisation of *cis-trans* proline bonds was found to be a rate-limiting step [9]. A second class of non-specific chaperones, the thiol-disulfide oxido-reductases (Dsbs) which catalyze the formation of disulfide bonds, has been shown previously to play a crucial role in the formation of disulfide bonds in heterologous proteins expressed in *E. coli* [10]. Homologues of *dsb* genes have recently been identified in *Pseudomonas aeruginosa*. Preliminary data on a deletion mutant of *dsbA* indicate an involvement in lipase folding (see [11]). Finally, overproduction of another type of non-specific periplasmic chaperone of *E. coli*, Skp, has been

shown to improve functional periplasmic expression of a slowly folding scFv [12]. Whether the effect of Skp is via its role in transporting lipopolysaccharide or via a direct interaction with the scFv fragments awaits further clarification. Homologues of this non-specific chaperone remain to be identified and tested in *Pseudomonas* species.

In addition to non-specific chaperones, the folding of a variety of extracellular proteins requires the action of specific chaperones. For example, the correct folding of lipases is mediated by the lipase-specific foldases (Lifs). It has been shown that folding of the lipase of *P. aeruginosa*, when expressed in *E. coli*, is dependent on the co-expression of the *P. aeruginosa lif* gene (F Rosenan, S Rösmann, KE Jaeger, personal communication). Interestingly, it was found that the amount of Lif could become limiting in an industrial *P. alcaligenes* strain upon overexpression of the endogenous lipase gene [2]. Different data have been published concerning the required level of Lif expression. On the one hand, *in vitro* refolding experiments with lipase and Lif suggest that a 1:1 ratio is optimal for the complete folding of the lipase [13]. On the other hand, in *P. alcaligenes* the Lif chaperone is required only in catalytic amounts [2]. Another set of specific chaperones, the propeptides of *P. aeruginosa* elastase and LasA protease, is secreted in association with their mature enzymes. Therefore, for the elastase propeptide, in addition to a role in folding the protein and in inhibiting its enzymatic activity in the periplasm, a function in targeting the protein to the secretion apparatus has been proposed [14•,15•]. A similar role can be rationalised for Lif as it becomes associated with the lipase in the periplasm [16]. It can be envisaged, however, that Lif is passed across the outer membrane in association with the lipase, holding it inactive during transport. In that case, the Lif protein would not be turned over in the periplasm and a 1:1 ratio of lipase to Lif expression would be required. On the other hand, the putative membrane anchor could fix the Lif protein to the inner membrane preventing transport of Lif over the outer membrane; in this case Lif would be required in catalytic amounts.

### Outer membrane translocation

In *P. aeruginosa*, the MTB of the GSP mediates the secretion of many unrelated proteins. Even though MTBs of GSPs have been found in many Gram-negative bacteria, secretion of heterologous proteins in non-related organisms often fails. For *Erwinia chrysanthemi* and *Erwinia caratovora*, it has been shown that a species-specific recognition of periplasmic intermediates by the secretion machinery occurs. In addition, the multicomponent outer membrane secretion machinery exhibits quite delicate internal interactions, as exchanging homologous components of MTBs of GSPs, in general, is not functional (see [6]). This implies that enhancement of the secretion capacity by overexpression of a single component of the machinery is not likely to be successful. *P. aeruginosa* $XcpQ_{GspD}$, a member of the secretin family, forms a homomultimeric, ring-shaped structure in the outer membrane. Overexpression of $XcpQ_{GspD}$ did not result in an increase in the amount of secreted proteins and was in fact lethal. It appeared that this lethality did not correlate with the proposed function of $XcpQ_{GspD}$ as a gated protein-conducting channel. It was concluded that, due to the lack of an XcpQ-specific chaperone, the complex is not correctly targeted to the outer membrane [17] and cell-death is caused by build-up of the protein. Although the MTB of the GSP allows for translocation of proteins across the outer membrane, many of its components are localised in the inner membrane (see [6]). $XcpY_{GspL}$, and $XcpX_{GspM}$ are required for mutual stabilisation [18]. When $XcpY_{GspL}$ is overexpressed, however, the excess of free $XcpY_{GspL}$ interferes with secretion, possibly due to non-functional interactions with other components of the secretion machinery [19]. Taken together, these data support the idea that a fine-tuned balance of the components of the type II secretion machinery is required for its proper functioning. In agreement with this notion, the 'phenotype enhancement method' identified the entire type II secretion machinery as a bottleneck for the secretion of *P. alcaligenes* lipase in the homologous host and a twofold increase in yield upon overexpression of the *xcp* gene cluster in an industrial host was reported [20•].

### Alternative secretion strategies

As mentioned before, the use of *E. coli* as a Gram-negative host for the production of heterologous proteins is hampered by the fact that it is not able to secrete proteins into the extracellular medium. As a result, overexpression of, for example, scFvs leads to the formation of insoluble, periplasmic aggregates. Rather then overproducing (non)specific chaperones, expression of these scFvs in L-form cells — lacking an intact outer membrane — of *E. coli* and *Proteus mirabilis*, resulted in the secretion of active products. It was proposed that the direct release of the translocated proteins into a large 'volume', the extracellular environment, lowered the 'local protein concentration', which favoured the folding of the peptides [21••]. L-form strains have been used successfully for the production of many other heterologous proteins in various Gram-negatives. Nevertheless, there is a perception in industry that use in large-scale fermentations probably will not work [22].

### Perspectives

Despite the fact that many unrelated proteins use the MTB of the GSP, the observed failure to secrete heterologous proteins suggests a species-specific recognition of the exoproteins. Thus, to use Gram-negatives as a general secretion host, the secretion determinant of the MTB of the GSP has to be identified. Because proteins using this pathway are translocated in a folded conformation, the secretion determinant could be a conformation signal. Evidence for the existence of such (a) secretion motif(s) has accumulated in recent years; however, neither on the amino acid level nor on the

structural level has any similarity pointing to a putative signal been found. It was therefore proposed that multiple independent signals might exist which are, each of them, required for subsequent recognition and/or secretion [6]. Following the recent reports that the specific chaperone of *P. aeruginosa* elastase is secreted in association with the enzyme, an alternative view has arisen. It was proposed that the structural secretion determinant could be located on the specific chaperones, rather than on the enzymes themselves [14•,15•]. Thus, it seems that periplasmic chaperones are the key for transport across the outer membrane.

The majority of data described above have been collected in the European Union project 'Pseudomonads as Enzyme Factories' [14•], which is focused on the expression of lipases in *P. aeruginosa* and *P. alcaligenes*. The results of the 'Pseudomonas Genome Project' (http://www.pseudomonas.com/) will facilitate the identification of yet unidentified folding catalysts, which can be subsequently engineered. The obtained knowledge could lead to a specialised host that folds Gram-negative proteins that require complex folding in a protected compartment (i.e. the periplasm) and subsequently sets free the folded protein in the extracellular medium via the MTB of the GSP. Ultimately, eukaryotic proteins requiring folding in a protected compartment might also become properly secreted via a *Pseudomonas* host.

## Gram-positive secretion hosts

### Engineering of the *B. subtilis* secretion machinery

Despite its high secretion capacity for homologous proteins, the protein secretion machinery of bacilli has certain limitations, which become evident when it is challenged with heterologous proteins — eukaryotic proteins with multiple disulfide bonds in particular. Bottlenecks in the secretion pathway of *B. subtilis* that have been reported so far relate to different stages in the secretion process. The first successful approaches to remove some of these secretion bottlenecks involved the overproduction of limiting secretion factors, such as PrsA, or the elimination of detrimental factors, such as proteases.

During the 'early' (cytosolic) stages in secretion, heterologous proteins may form insoluble aggregates in the cytoplasm, which is, most probably, caused by limited activity of chaperones. For example, the secretion of an antidigoxin scFv, which has the tendency to accumulate in inclusion bodies, was shown to be improved by about 60% through the concerted overproduction of the cytosolic GroEL-GroES and DnaK-DnaJ-GrpE chaperone machineries [23••]. Whether the aggregation of secretory proteins in the cytoplasm also reflects a limited transport capacity of the preprotein translocase in the membrane is presently not known.

During or shortly after translocation across the membrane, signal peptidases can be limiting factors in preprotein processing. For example, if preproteins fold rapidly upon translocation (i.e. before the signal peptide has been removed), they can become incompetent for processing. This problem can be prevented by signal peptidase overproduction (see [24]). Alternatively, signal peptidases can also interfere by an unknown mechanism with efficient preprotein processing under conditions of high-level overproduction of secretory proteins, as illustrated by the observation that the disruption of the *sipS* gene for one of the five signal peptidases of *B. subtilis*, resulted in highly increased rates of processing of an α-amylase precursor (see [25,26••]).

Finally, late stages in the secretion process, involving the folding of mature proteins and cell-wall passage, can be potential secretion bottlenecks. Firstly, the lipoprotein PrsA, a putative PPI, sets a limit to the high-level secretion of certain proteins, such as α-amylases and proteases from Gram-positive eubacteria, as PrsA is required for their folding into a protease-resistant conformation upon translocation (see [27]). Notably, PrsA overproduction did not only improve the secretion of eubacterial proteins, but also that of engineered eukaryotic proteins, such as scFv [23••]. Secondly, it has been suggested that the cell wall forms a barrier for at least one secreted heterologous protein, human serum albumin [28]. This could be due to the fact that the cell wall is relatively thick (10–50 nm) and contains a high concentration of immobilised negative charge (e.g. teichoic or teichuronic acids; see [29]); thus, proteins with a net positive charge might be retained in the wall. Finally, at all stages in the secretion process, heterologous proteins are vulnerable to degradation by proteases. Recent studies suggest that, in addition to at least six secreted proteases (see [23••]), proteases residing at the membrane–cell-wall interface can be particularly problematic in this respect [30,31]. For example, it was shown that the wall-bound serine protease CWBP52, specified by the *wprA* gene [32], is active at the site of preprotein translocation [33•], and that CWBP52 depletion results in increased yields of secreted α-amylase [34•]. Thus, proteases of the latter type are likely to be involved in the quality control of secreted proteins by removing incorrectly folded proteins in order to prevent jamming of the secretory pathway.

### Perspectives

The observations described above clearly point out that the optimisation of the secretion of individual heterologous proteins is likely to require engineered *B. subtilis* strains in which specific secretion bottlenecks have been removed. Fortunately, our knowledge of the secretion pathway of *B. subtilis* and its potential bottlenecks is rapidly increasing. This can be attributed, to a large extent, to the availability of the complete genome sequence of *B. subtilis*, which, for example, allowed the identification of the large majority of potentially detrimental proteases of *B. subtilis*. Thus, it is anticipated that dedicated *B. subtilis* host strains for the secretion of a wide range of

heterologous proteins at commercially significant levels will soon become available. Based on the recent identification of thiol-disulfide oxido-reductases of *B. subtilis* [P3] (A Bolhuis, S Bron, J-M van Dijl, unpublished data), we are confident that this will even apply to heterologous proteins with multiple disulfide bonds.

## Conclusions

The increased knowledge of the bacterial secretion process has lead to the identification of several bottlenecks for the production of heterologous proteins. A rational selection and design of an expression host should be based on the specific properties of the subject protein. For complex proteins with multiple disulfide bonds, the periplasm equipped with non-specific and optionally specific chaperones can provide an ideal folding compartment. The outer membrane transport capacity can be enhanced by overproducing the components of the MTB of the GSP. Alternatively, the outer membrane barrier can be circumvented using L-forms.

Secretion from *Bacillus* cells results in the highest yield and easiest recovery provided that the protein can be folded sufficiently fast into a protease-resistant conformation after passage of the translocase. The engineering of extracellular foldases, such as PPIs and Dsbs, and the elimination of cell-wall-associated proteases may lead to successful secretion of more complex proteins from *Bacillus*.

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