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Review Article

Recombinant protein secretion by *Bacillus subtilis* and *Lactococcus lactis*: pathways, applications, and innovation potential

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Secreted recombinant proteins are of great significance for industry, healthcare and a sustainable bio-based economy. Consequently, there is an ever-increasing need for efficient production platforms to deliver such proteins in high amounts and high quality. Gram-positive bacteria, particularly bacilli such as *Bacillus subtilis*, are favored for the production of secreted industrial enzymes. Nevertheless, recombinant protein production in the *B. subtilis* cell factory can be very challenging due to bottlenecks in the general (Sec) secretion pathway as well as this bacterium's intrinsic capability to secrete a cocktail of highly potent proteases. This has placed another Gram-positive bacterium, *Lactococcus lactis*, in the focus of attention as an alternative, non-proteolytic, cell factory for secreted proteins. Here we review our current understanding of the secretion pathways exploited in *B. subtilis* and *L. lactis* to deliver proteins from their site of synthesis, the cytoplasm, into the fermentation broth. An advantage of this cell factory comparison is that it identifies opportunities for protein secretion pathway engineering to remove or bypass current production bottlenecks. Noteworthy new developments in cell factory engineering are the mini-*Bacillus* concept, highlighting potential advantages of massive genome minimization, and the application of thus far untapped 'non-classical' protein secretion routes. Altogether, it is foreseen that engineered lactococci will find future applications in the production of high-quality proteins at the relatively small pilot scale, while engineered bacilli will remain a favored choice for protein production in bulk.

Introduction

To thrive and survive in different ecological niches, bacteria secrete a wide range of different proteins. This allows them to take optimal advantage of their habitat, as exemplified by the secretion of proteases that facilitate the acquisition of peptides and amino acids, be it in the soil through the degradation of organic matter or in the industrial processing of milk for the production of cheese [1]. Gram-positive bacteria are known for their intrinsic capacity to secrete proteins directly into the extracellular milieu [2]. This relates to the relatively simple structure of their cell envelope where, in the most elementary form, the membrane is surrounded by a relatively thick, porous cell wall composed of peptidoglycan and several other polymers. Accordingly, some proteins can pass the cell wall directly upon membrane translocation, while the extracellular release of others may depend on the activity of specific enzymes, such as cell wall hydrolases [3]. In general, the secretion of proteins from the cytoplasm to the extracellular milieu is not a spontaneous process. It requires membrane channels and an intricate machinery that converts metabolic energy into a force that drives proteins through the membrane. Furthermore, many integral membrane proteins are inserted into the bacterial cytoplasmic membrane via the same export pathway that is followed

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by secreted proteins. Consequently, protein translocation across the cytoplasmic membrane is an essential process [4].

A Gram-positive bacterium that is well known for its high capacity to secrete proteins is *Bacillus subtilis*. In nature, *B. subtilis* flourishes in the challenging niches provided by the soil and plant rhizosphere, where it secretes a cocktail of different degradative enzymes to obtain nutrients (e.g. sugars, amino acids, phosphate and metal ions), and to defend itself from chemical and biological insults [5]. Due to its well-developed and highly efficient secretion machinery, *B. subtilis* has also become a popular biotechnological ‘cell factory’ for the bulk production of commercially relevant secreted proteins, in particular enzymes such as proteases, amylases and xylanases [6–8]. Industrial enzymes are used in many different markets including personal care, food and beverages, detergents, textiles, animal feed, chemicals and biofuels. They permeate every aspect of our daily life and the markets are consequently large. Today there is a need for new, improved and more versatile enzymes in order to develop more novel, sustainable and economically competitive production processes. Accordingly, the secretion system of *B. subtilis* and the full complement of secreted proteins, the ‘secretome’, have been intensely investigated. Importantly, the studies on *B. subtilis* also provided new insights into the secretion pathways present in other Gram-positive bacteria, especially bacilli, lactic acid bacteria, streptococci and staphylococci [9,10].

Gram-positive bacteria lack the outer membrane that is present in Gram-negative bacteria, such as *Escherichia coli*. This absence not only simplifies the secretion process, but it also has the great advantage that Gram-positive bacteria lack lipopolysaccharides, also called endotoxins [11]. To avoid the barrier imposed by the outer membrane, *E. coli*-based expression systems often aim at protein production in the cytoplasm. This allows the accumulation of massive amounts of product in the cytoplasm but, at the same time, it significantly complicates the downstream processing of the produced proteins. This is further convoluted by the fact that proteins overproduced in the cytoplasm often aggregate, forming so-called inclusion bodies from which the product can only be recovered upon cell disruption and treatment with strong denaturing agents, such as urea. Conversely, the downstream processing of proteins secreted into the fermentation broth of Gram-positive bacteria is generally easy, non-denaturing and cost-effective [11]. Amongst the Gram-positive bacteria, *B. subtilis* and related bacilli offer the additional advantage that they can be readily fermented at large scale using cheap carbon sources, which adds to the cost-effectiveness. This is important as industrial enzymes, in contrast with biopharmaceuticals, are marketed at relatively low prices [11]. A particular advantage of *B. subtilis* is that, due to the absence of toxins, products from this cell factory have obtained the Generally Recognized as Safe (GRAS) status from the United States Food and Drug Administration (FDA). In addition, *B. subtilis* is on the list of organisms with Qualified Presumption of Safety (QPS) status, assembled by the European Food Safety Authority (EFSA) [11]. This makes *B. subtilis* very attractive as a cell factory, not only for food enzymes, but perhaps even more so for biotherapeutics that are used to treat an increasingly wide range of serious diseases.

Despite all the advantages, production of secreted recombinant proteins in *B. subtilis* is frequently challenging. In particular, the fact that *B. subtilis* naturally secretes multiple proteases can interfere with the production of proteins that are sensitive to degradation [7,12]. Even though strains lacking up to ten different proteases have been developed, this problem has still not been completely overcome, as detailed in Supplementary Table S1. Moreover, various other secretion bottlenecks are also known to exist at the levels of membrane targeting, translocation and post-translocational protein folding [11]. Thus, while *B. subtilis* is overall a highly attractive cell factory that is intensively exploited in the industry [13], there is room for additional, preferably Gram-positive, bacterial platforms for secretory protein production in which the aforementioned disadvantages can be circumvented. One such platform could be *Lactococcus lactis*, a food-grade Gram-positive bacterium which is, thus far, used mostly in the dairy industry.

Sec-dependent protein translocation pathway

In Gram-positive bacteria like *B. subtilis* and *L. lactis*, protein secretion is mainly facilitated by the so-called Sec-dependent protein translocation pathway. To specifically transport proteins from the ribosome in the cytosol to a translocation channel in the cytoplasmic membrane, the respective proteins are synthesized with N-terminal signal peptides (SPs). The different known SPs are variable in length and show little amino acid sequence similarity [14]. Nevertheless, they show structural conservation as they invariably consist of (i) a positively charged N-terminal region with lysine or arginine residues and, incidentally, histidine residues, (ii) a central H-region, consisting of mostly hydrophobic residues, with the potential to adopt an α -helical conformation, and (iii) a hydrophilic C-region with a type I signal peptidase recognition site including the Ala-x-Ala consensus motif. The C-region of SPs from secreted proteins has a β -stranded conformation to allow recognition and subsequent cleavage C-terminally of the Ala-x-Ala motif by multiple type I signal peptidases (SipS-SipW) in *B. subtilis* [9,14,15], or by the unique type I signal peptidase SipL in *L. lactis* [16]. In contrast, the SPs of lipoproteins, which remain attached to the membrane by a diacyl-glycerol

modification, contain a consensus Leu-x-x-Cys motif for recognition by type II signal peptidase (LspA), where cleavage takes place N-terminally of the strictly conserved Cys residue [17]. While many different SPs are applied for protein production in *B. subtilis* (Supplementary Table S1), the preferred SP for protein production in *L. lactis* is derived from the major secreted lactococcal protein Usp45 (Supplementary Table S2).

The Sec-dependent secretion machinery can be divided into six sections: (i) cytosolic factors like chaperones that may keep exported proteins in an unfolded state prior membrane translocation (i.e. CsaA, GroEL/ES, DnaK, DnaJ, GrpE, trigger factor) or that guide the exported proteins to the membrane (i.e. SRP, FtsY); (ii) the precursor protein translocase consisting of the ATP-dependent translocation motor (i.e. SecA) cooperating with the membrane-embedded translocation channel (i.e. SecYEG, SecDF-YajC, SpoIIIJ/YqjG/YidC); (iii) the aforementioned signal peptidases that cleave the SP from the precursor protein during the translocation process, especially SipS-SipW [9,14,15] in *B. subtilis* and SipL in *L. lactis* [16]; (iv) SP peptidases that remove remnants of SPs from the cell membrane (i.e. SppA, TepA, RasP in *B. subtilis*; RasP and TepA in *L. lactis*); (v) membrane-associated folding catalysts to ensure proper folding of the translocated proteins at the *trans*-side of the membrane (i.e. PrsA, BdbB-D in *B. subtilis*; PrtM, PmpA in *L. lactis*); and (vi) a range of different exported proteases that drive the quality control of secretory proteins (i.e. HtrA-C, WprA, PrsW [in the cell envelope] and AprE, Bpr, Epr, Mpr, NprB, NprE Vpr [extracellular] in *B. subtilis*; HtrA, PrtP in the cell envelope of *L. lactis*). The similarities and differences in the secretion machinery of both organisms are schematically represented in Figure 1.

The widely conserved mechanisms of Sec-dependent protein secretion have been extensively reviewed in recent years [2,14,18,19]. Therefore, they are not detailed in the present review. Instead, we focus attention on studies that provide clues for further optimization of recombinant protein production by the microbial cell factories *B. subtilis* and *L. lactis*.

Strain improvement

The earliest efforts to optimize *B. subtilis* as a cell factory for secreted heterologous proteins were focused on the identification and subsequent removal of extracellular proteases that are, due to their intrinsic function, responsible for product loss. This led to the successive construction of frequently applied protease mutant strains like DB104 ($\Delta aprE$, $\Delta nprE$), WB600 ($\Delta nprE$, $\Delta aprE$, Δepr , Δbpr , Δmpr , $\Delta nprB$), WB700 (WB600 Δvpr) and WB800 (WB700 $\Delta wprA$) [13,20–22]. More recently the BRB strain collection was constructed, which includes strains lacking up to ten extracytoplasmic and/or secreted proteases. Some of the latter strains lack the proteases deleted from the WB800 strain *plus* HtrA and HtrB [13]. While the WB600-WB800 strains still include an erythromycin resistance marker, all BRB strains have ‘clean’ deletions of the respective protease genes. The WB800 strain is, to date, the most frequently used *B. subtilis* strain for production and secretion of heterologous proteins (Supplementary Table S1). Noteworthy examples of the use of protease mutants include the production of a thermostable β -1,3-1,4-glucanase from *Clostridium thermocellum* [23], and pharmaceutical proteins, such as single-chain antibodies [24] or human interleukin-3 [21].

Major efforts have also been made to tune the protein secretion machinery of *B. subtilis* for heterologous protein production. For example, it was shown at the laboratory scale that overexpression of the signal peptidase SipS [25,26], or the peptidyl-prolyl *cis/trans* isomerase and foldase PrsA with or without the chaperone DnaK [27,28] could enhance the secretion of particular proteins. Besides this, it was recently shown that the co-expression of heterologous PrsA proteins with amylases from the same origin can enhance the secretory production of the respective amylase, thereby reducing secretion stress significantly [29]. On the other hand, improved export rates of the α -amylase AmyQ could also be achieved by deleting the signal peptidase genes *sipS* or *sipU* [30]. The latter observation could be explained by the fact that different *B. subtilis* signal peptidases compete for binding the same precursor molecule, but may have different catalytic efficiencies. Elimination of less effective redundant signal peptidases would thus have a beneficial effect on enzyme secretion. Furthermore, the co-expression of heterologous secretion machinery components was shown to be potentially beneficial for enhancing protein secretion in *B. subtilis*, as exemplified with SecB of *E. coli* [31] and the staphylococcal thiol-disulfide oxidoreductase DsbA [32]. More extensive engineering approaches have also been applied to improve the capacity of the Sec machinery of *B. subtilis*. In one case, this involved a deletion in the C-terminal linker domain of SecA, which led to 2.2-fold enhanced secretion of human interferon- α 2b [33]. In another case, *B. subtilis* SecA was provided with the SecB-binding 32 C-terminal residues of *E. coli* SecA to enhance the effects of SecB co-expression. This allowed the improved secretion of model proteins derived from *E. coli*, in particular a mutant of the maltose-binding protein (MalE11) and the alkaline phosphatase PhoA [34]. Possibly the most noticeable improvement was achieved by overexpressing the intramembrane protease RasP, which enhanced the secretion of two enzymes that were difficult to produce approx. 2.5- to 10-fold in industrial fermentation-mimicking conditions. This result suggests that the activity of RasP, which is cleaving peptides in the

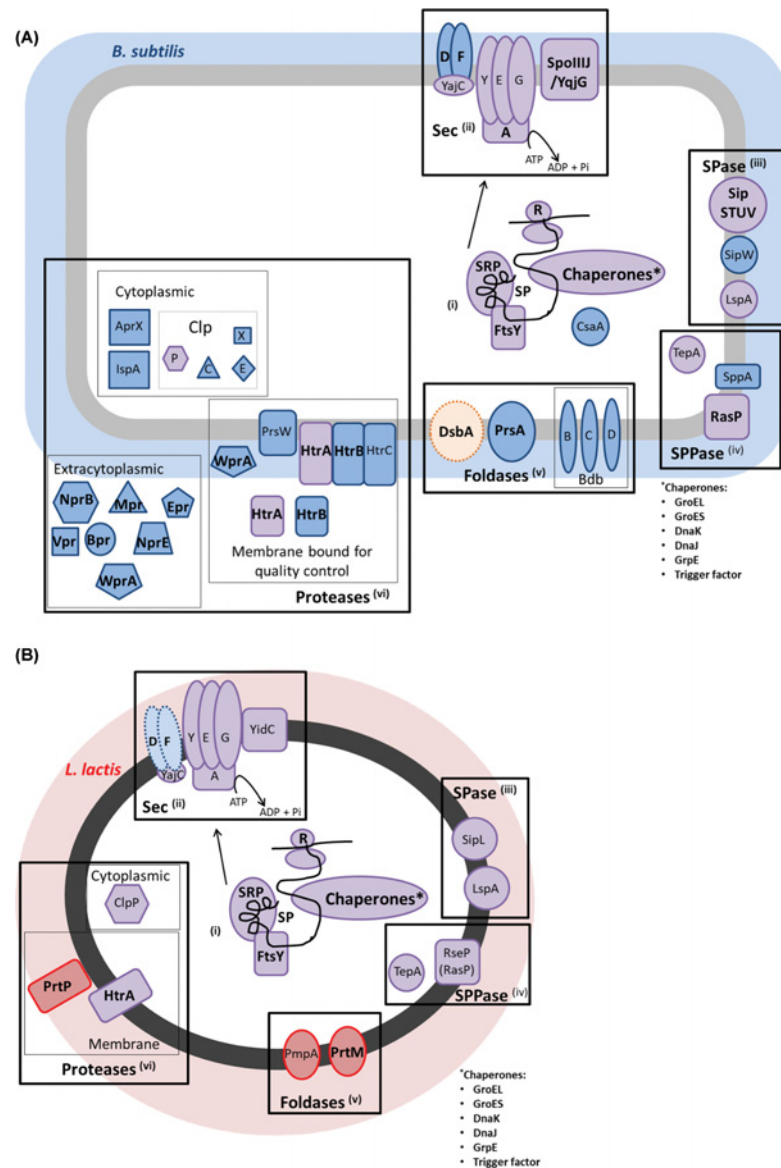


Figure 1. Comparison of the main components of the Sec-dependent protein translocation machinery of *B. subtilis* and *L. lactis*

Overview of components of the Sec-dependent protein export machinery of (A) *B. subtilis* and (B) *L. lactis*. (i) Upon ribosomal (R) translation, exported membrane and secretory precursor proteins will be specifically targeted to the Sec pathway by means of their SP. In addition, these proteins will be recognized by cytoplasmic chaperones and targeting factors, including SRP/FtsY, CsaA and SecA, which may keep the exported proteins in a translocation-competent state. (ii) The Sec translocase consists of the ATP-dependent SecA motor protein and channel components SecY, SecE and SecG. The Sec translocase of *B. subtilis* also includes the proton-motive force-dependent translocation motor SecDF–YajC. Particular membrane proteins require the homologous SpoIIJ/YajG or YidC insertases for membrane biogenesis. (iii) Shortly after or during the translocation process, the precursor protein is cleaved by one of the type I signal peptidases (SPase; SipS–V, SipL or SipW), or the type II signal peptidase LspA. (iv) TepA, SppA, and RasP have been implicated in the degradation of cleft SPs. (v) The folding of various exported proteins is dependent on the action of the folding catalysts PrsA, PmpA, PrtM, or BdbB–D. (vi) HtrA, HtrB, HtrC, WprA and PrsW are implicated in quality control of the membrane and secretory proteins. Of note, HtrA and HtrB of *B. subtilis* have a dual localization in the membrane and growth medium. Extracellular proteases AprE, Bpr, Epr, Mpr, NprB, NprE and Vpr of *B. subtilis* can have roles in the degradation of secreted proteins. HtrA and PrtP are the major proteases of *L. lactis*. Intracellular proteases ClpC–ClpP, ClpE–ClpP, ClpX–ClpP, AprX, IspA and TepA may be involved in the degradation of mis-targeted exported proteins. Secretion machinery components that have been engineered for improved protein secretion are marked in bold. Of note, staphylococcal DsbA in *B. subtilis* and *B. subtilis* SecDF in *L. lactis* were heterologously expressed in *B. subtilis* and *L. lactis*, respectively (dotted line).

plane of the membrane, helps to remove remnant SPs or precursor proteins that perturb the membrane, which would render the overall protein secretion less effective [35]. Altogether, the previously published observations show that engineering of the Sec machinery for enhanced protein secretion is a feasible approach. However, probably not one single engineering scenario will resolve all encountered bottlenecks. This relates to the fact that different secretion bottlenecks are encountered for different heterologous proteins [36,37].

A relatively new approach for enhancing the protein secretion capacity of *B. subtilis* is genome engineering, which has become feasible due to the identification of all essential genes of this bacterium and the availability of effective tools to delete large parts of the chromosome [38]. In doing so, strain PG10 was developed which lacks approx. 36% of the *B. subtilis* genome [39]. In fact, the PG10 strain represents the most minimized *Bacillus* chassis known to date. This ‘mini-*Bacillus*’ was shown to be favorable for the secretion of ‘difficult-to-produce proteins’ [38]. In particular, strain PG10 overcomes several bottlenecks in the production of staphylococcal antigens, which seems to relate to strongly reduced production of proteases and an increased translational efficiency.

Recent studies have demonstrated that *L. lactis* can also be exploited in the production of various protease-sensitive proteins, including potential antigens for vaccination [40–43]. Intriguingly, despite the important role of proteolysis in the degradation of casein during cheese ripening, *L. lactis* produces no more than three major proteases that can have an impact on protein production. The major secreted protease needed for cheese production is PrtP, but this plasmid-encoded enzyme is dispensable for growth of *L. lactis* on media other than milk [44,45]. *L. lactis* possesses two additional house-keeping proteases, namely the extracytoplasmic protease HtrA and the cytoplasmic protease ClpP. Deletion of the *htrA* gene results in highly reduced proteolysis of various recombinant secreted proteins (Supplementary Table S2), showing a major function in extracytoplasmic protein turnover [46–48]. In particular, Sriraman et al. showed that the degradation of secreted streptokinase was substantially reduced when the protein was produced in a strain lacking *htrA* [49]. In contrast, deletion of the *clpP* gene does not further enhance extracellular protein production, as exemplified with the secreted nuclease Nuc of *Staphylococcus aureus* and the human papillomavirus E7 protein fused to Nuc [46]. The notion that the low proteolytic activity of *L. lactis* is advantageous was further supported by the observation that several proteinaceous antigens from *S. aureus* can be produced and secreted by *L. lactis*, but not in wildtype *B. subtilis* or mutant *B. subtilis* strains lacking particular protease genes. This was only recently shown to be possible in the aforementioned genome-engineered ‘mini-*Bacillus*’ strain PG10 [38]. Further, similar to the situation in *Bacillus*, it seems that also the secretion capacity of *L. lactis* can be enhanced through the introduction of heterologous Sec components. This was exemplified with SecDF, which is absent from wild-type *L. lactis* strains. Nonetheless, it was shown that protein export in this bacterium can be enhanced to some extent by heterologous expression of *secDF* from *B. subtilis* [50]. Also, the overexpression of the extracytoplasmic folding catalyst PmpA, a homologue of *B. subtilis* PrsA, allowed improved production of a lipase from *Staphylococcus hyicus* [51]. A particularly noteworthy *L. lactis* strain improvement was achieved by deleting the gene for the major autolysin AcmA, leading to substantially reduced levels of cell lysis and, therefore, lowered levels of contaminating cytoplasmic proteins. Using an *htrA acmA* double mutant, i.e. *L. lactis* strain PA1001, various staphylococcal antigens were stably produced and secreted into the growth medium [41,43,47].

Despite the attractive traits of *L. lactis* for recombinant protein production, it has to be noted that the overall production yields achieved with *L. lactis* have so far remained significantly lower than those achieved with *B. subtilis* [47]. At the laboratory scale, the difference in yields is approx. 100-fold with *B. subtilis* secreting proteins in the gram per liter range. Under optimized fermentation conditions *B. subtilis* can easily produce 25 grams of protein per liter culture [6,8,11]. Although there have been no systematic comparisons for the secretion kinetics and final production levels of the same proteins in *B. subtilis* and *L. lactis*, the apparent difference in productivity may be attributable to multiple factors. First, *B. subtilis* can be grown to much higher cell densities than *L. lactis*. Further, *L. lactis* has the tendency to acidify its growth medium due to lactate production, which requires strict pH control and may set a limit to growth. Importantly, the secretory capacity of *L. lactis* appears lower, which may relate to differences in the machinery for protein secretion as detailed above and evolutionary pressures. While the secretion machinery of *L. lactis* was geared towards growth in milk [52], *B. subtilis* has evolved in environments with mostly polymeric carbon sources that require extracellular degradation before they can be utilized by the bacterium. Still, the fact that *B. subtilis* secretes more proteins than *L. lactis* is not necessarily related to the capacity of the respective secretion machinery. In particular, protein production levels in the gram per liter range have been obtained with highly optimized *B. subtilis* strains while, so far, less efforts have been undertaken to optimize the productivity of *L. lactis*.

A novel ‘non-classical protein secretion’ approach

A common phenomenon among bacteria is the release of cytoplasmic proteins into the growth medium [9,10,53–55]. The mechanisms underlying the release of these ‘extracellular cytoplasmic proteins’ (ECPs) [56] is not clearly defined,

but it has been associated with as yet unidentified protein export pathways, the synthesis of autolysins, prophage activity, the production of cytolytic toxins and surfactants, shedding of membrane vesicles, and cell death [57–61]. The level of ECPs that can be detected in *B. subtilis* is inversely related to protease production with protease-deficient strains showing high ECP levels, and protease-overproducing strains showing low ECP levels [54,57]. Thus, the numbers of identified ECPs may range between ~24 and a few hundred proteins, depending on the *B. subtilis* strain and the sensitivity of the applied proteomics approach for extracellular protein detection [9,57]. For *L. lactis*, at least four ECPs have been reported [62]. In pathogens the non-classically secreted proteins can serve important biological functions, for example in the bacterial adherence to substrates or host cells and tissues, or in biofilm formation [63–68]. This raises the intriguing question whether non-classical protein secretion can also be useful for biotechnological applications. Indeed, in recent years several publications have reported on the successful non-classical secretion of homologous proteins, and even heterologous bacterial or eukaryotic proteins in *B. subtilis*, which bypassed the SP- and Sec-dependent protein export pathway. Instead, the target proteins were fused to a D-psicose 3-epimerase from *Ruminococcus sp.* [69]. Proteins thus produced were effectively recovered from the growth medium, and the published data suggest that this secretion route could be independent of cell lysis or extracellular membrane vesicle formation [70,71].

Conclusion

The present overview summarizes the pros and cons of the Gram-positive bacterial cell factories *B. subtilis* and *L. lactis*. Based on the available data, we conclude that *B. subtilis* and closely related bacilli are most suitable for the bulk production of recombinant proteins. However, *B. subtilis* is highly proteolytic which represents a serious drawback as this may lead to a loss of product and/or the accumulation of cleaved product derivatives. This can be overcome by the deletion of protease genes, but the resulting strains are oftentimes more sensitive to autolysis, leading to increased amounts of contaminating cytoplasmic proteins in the fermentation broth. The increased sensitivity for lysis is probably one of the main reasons why multiple protease-deficient *Bacillus* strains are not frequently used in the industrial setting. On the other hand, the possible use of *L. lactis* is increasingly explored for secreted protein production. Although the amounts of protein that are produced by *L. lactis* are significantly lower than is generally the case for *B. subtilis* (Supplementary Tables S1 and S2), *L. lactis* has one big advantage over *Bacillus*, namely the nearly complete absence of protease activity once the *prtP* and *htrA* protease genes have been eliminated. Strains lacking these two genes allow enhanced secretory production of several recombinant proteins. Another advantage of the *L. lactis* protease-deficient strains is the low level of autolysis, in particular when the *acmA* gene encoding for the major autolysin is deleted. Engineering of autolysins in protease-deficient *Bacillus* strains was not yet systematically explored, but it could definitely lead to improved strains that are less prone to cell lysis. In this context, it is important to bear in mind that the industrial *Bacillus* strains that produce secretory proteins in very high quantities are, in general, strains that have a long history of optimization with respect to their fermentation properties and protein production. In contrast, protein production in *L. lactis* has only just started to be explored, which leaves open many opportunities for further strain improvements. Nonetheless, based on the current species-specific differences in the capacity for protein secretion, we conclude that *B. subtilis* and related bacilli are the more suitable platforms for protein production in bulk amounts. On the other hand, *L. lactis* appears to be most useful for the pilot production of recombinant proteins that are highly susceptible to degradation, such as antigens for vaccine production. It seems likely that the latter can also be achieved with engineered *Bacillus* species, such as the aforementioned mini-*Bacillus*, but this will require a better understanding of the interplay between the proteolytic and autolytic systems of these bacteria.

Summary

- Gram-positive bacteria secrete proteins directly into the fermentation broth, making them particularly suited as cell factories for recombinant protein production.
- *B. subtilis* is best-suited for recombinant protein secretion in bulk.
- *L. lactis* can be applied to produce high-quality proteins at pilot scale.
- Secretory pathway engineering and genome engineering open up new avenues for recombinant protein production in microbial cell factories.
- There is benefit in exploring ‘non-classical’ protein secretion routes for recombinant protein production.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

J.N. drafted the manuscript. J.M.v.D. and G.B. supervised the project and edited the manuscript. J.N., J.M.v.D. and G.B. revised the manuscript and approved the final version.

Abbreviations

ECP, extracellular cytoplasmic protein; SP, signal peptide.

References

- 1 Richardson, A.R., Somerville, G.A. and Sonenshein, A.L. (2015) Regulating the intersection of metabolism and pathogenesis in Gram-positive bacteria. *Microbiol. Spectr.* **3**, 129–165, <https://doi.org/10.1128/microbiolspec.MBP-0004-2014>
- 2 Anne, J., Economou, A. and Bernaerts, K. (2017) Protein secretion in Gram-positive bacteria: from multiple pathways to biotechnology. *Curr. Top. Microbiol. Immunol.* **404**, 267–308, https://doi.org/10.1007/82_2016_49
- 3 Forster, B.M. and Marquis, H. (2012) Protein transport across the cell wall of monoderm Gram-positive bacteria. *Mol. Microbiol.* **84**, 405–413, <https://doi.org/10.1111/j.1365-2958.2012.08040.x>
- 4 Yuan, J., Zweers, J.C., van Dijk, J.M. and Dalbey, R.E. (2010) Protein transport across and into cell membranes in bacteria and archaea. *Cell Mol. Life Sci.* **67**, 179–199, <https://doi.org/10.1007/s00018-009-0160-x>
- 5 Earl, A.M., Losick, R. and Kolter, R. (2008) Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol.* **16**, 269–275, <https://doi.org/10.1016/j.tim.2008.03.004>
- 6 Van Dijk, J.M. and Hecker, M. (2013) *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microb. Cell Fact.* **12**, 3, <https://doi.org/10.1186/1475-2859-12-3>
- 7 Harwood, C.R. and Cranenburgh, R. (2007) *Bacillus* protein secretion: an unfolding story. *Trends Microbiol.* **16**, 73–79, <https://doi.org/10.1016/j.tim.2007.12.001>
- 8 Yoshida, K. and Van Dijk, J.M. (2020) Engineering *Bacillus subtilis* cells as factories: enzyme secretion and value-added chemical production. *Biotechnol. Bioproc. Eng.* **25**, 872–885, <https://doi.org/10.1007/s12257-020-0104-8>
- 9 Tjalsma, H., Antelmann, H., Jongbloed, J.D.H., Braun, P.G., Darmon, E. et al. (2004) Proteomics of protein secretion by *Bacillus subtilis*: separating the ‘secrets’ of the secretome. *Microbiol. Mol. Biol. Rev.* **68**, 207–233, <https://doi.org/10.1128/MMBR.68.2.207-233.2004>
- 10 Sibbald, M.J.J.B., Ziebandt, A.K., Engelmann, S., Hecker, M., De Jong, A., Hamsen, H.J.M. et al. (2006) Mapping the pathway to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol. Biol. Rev.* **70**, 755–788, <https://doi.org/10.1128/MMBR.00008-06>
- 11 Hohmann, H.P., Van Dijk, J.M., Krishnappa, L. and Prágai, Z. (2016) Host organisms: *Bacillus subtilis*. In *Industrial Biotechnology* (Wittmann, C. and Liao, J.C., eds), pp. 183–220, Wiley-VCH Verlag, GmbH
- 12 Pohl, S. and Harwood, C.R. (2010) Heterologous protein secretion by *Bacillus* species from the cradle to the grave. *Adv. Appl. Microbiol.* **73**, 1–25, [https://doi.org/10.1016/S0065-2164\(10\)73001-X](https://doi.org/10.1016/S0065-2164(10)73001-X)
- 13 Pohl, S., Bhavsar, G., Hulme, J., Bloor, A.E., Misirli, G., Leckenby, M.W. et al. (2013) Proteomic analysis of *Bacillus subtilis* strains engineered for improved production of heterologous proteins. *Proteomics* **13**, 3298–3308, <https://doi.org/10.1002/pmic.201300183>
- 14 Dalbey, R.E., Wang, P. and van Dijk, J.M. (2012) Membrane proteases in the bacterial protein secretion and quality control pathway. *Microbiol. Mol. Biol. Rev.* **76**, 311–330, <https://doi.org/10.1128/MMBR.05019-11>
- 15 Tjalsma, H., Bolhuis, A., Jongbloed, J.D.H., Bron, S. and Van Dijk, J.M. (2000) Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* **64**, 515–547, <https://doi.org/10.1128/MMBR.64.3.515-547.2000>
- 16 Morello, E., Bermudez-Humaran, L.G., Llull, D., Sole, V., Miraglio, N., Langella, P. et al. (2008) *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. *J. Mol. Microbiol. Biotechnol.* **14**, 48–58, <https://doi.org/10.1159/000106082>
- 17 Tjalsma, H., Kontinen, V.P., Pragai, Z., Wu, H., Meima, R., Venema, G. et al. (1999) The role of lipoprotein processing by signal peptidase II in the Gram-positive eubacterium *Bacillus subtilis*. Signal peptidase II is required for the efficient secretion of alpha-amylase, a non-lipoprotein. *J. Biol. Chem.* **274**, 1698–1707, <https://doi.org/10.1074/jbc.274.3.1698>
- 18 Tsirigotaki, A., De Geyter, J., Sostaric, N., Economou, A. and Karamanou, S. (2017) Protein export through the bacterial Sec pathway. *Nat. Rev. Microbiol.* **15**, 21–36, <https://doi.org/10.1038/nrmicro.2016.161>
- 19 Schneewind, O. and Missiakas, D. (2014) Sec-secretion and sortase-mediated anchoring of proteins in Gram-positive bacteria. *Biochim. Biophys. Acta* **1843**, 1687–1697, <https://doi.org/10.1016/j.bbamcr.2013.11.009>
- 20 Kawamura, F. and Doi, R.H. (1984) Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. *J. Bacteriol.* **160**, 442–444, <https://doi.org/10.1128/JB.160.1.442-444.1984>

- 21 Westers, L., Dijkstra, D.S., Westers, H., van Dijk, J.M. and Quax, W.J. (2006) Secretion of functional human interleukin-3 from *Bacillus subtilis*. *J. Biotechnol.* **123**, 211–224, <https://doi.org/10.1016/j.jbiotec.2005.11.007>
- 22 Westers, L., Westers, H., Zanen, G., Antelmann, H., Hecker, M., Noone, D. et al. (2008) Genetic or chemical protease inhibition causes significant changes in the *Bacillus subtilis* exoproteome. *Proteomics* **8**, 2704–2713, <https://doi.org/10.1002/pmic.200800009>
- 23 Luo, Z., Gao, Q., Li, X. and Bao, J. (2014) Cloning of LicB from *Clostridium thermocellum* and its efficient secretive expression of thermostable beta-1,3-1,4-glucanase. *Appl. Biochem. Biotechnol.* **173**, 562–570, <https://doi.org/10.1007/s12010-014-0863-9>
- 24 Wu, X.C., Ng, S.C., Near, R.I. and Wong, S.L. (1993) Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Biotechnology* **11**, 71–76, <https://doi.org/10.1038/nbt0193-71>
- 25 van Dijk, J.M., de Jong, A., Vehmaanpera, J., Venema, G. and Bron, S. (1992) Signal peptidase I of *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. *EMBO J.* **11**, 2819–2828, <https://doi.org/10.1002/j.1460-2075.1992.tb05349.x>
- 26 Bolhuis, A., Sorokin, A., Azevedo, V., Ehrlich, S.D., Braun, P.G., De Jong, A. et al. (1996) *Bacillus subtilis* can modulate its capacity and specificity for protein secretion through temporally controlled expression of the *sipS* gene for signal peptidase I. *Mol. Microbiol.* **22**, 605–618, <https://doi.org/10.1046/j.1365-2958.1996.d01-4676.x>
- 27 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, <https://doi.org/10.1111/j.1365-2958.1993.tb01616.x>
- 28 Chen, J., Fu, G., Gai, Y., Zheng, P., Zhang, D. and Wen, J. (2015) Combinatorial Sec pathway analysis for improved heterologous protein secretion in *Bacillus subtilis*: identification of bottlenecks by systematic gene overexpression. *Microb. Cell Fact.* **14**, 92–105, <https://doi.org/10.1186/s12934-015-0282-9>
- 29 Quesada-Ganuza, A., Antelo-Varela, M., Mouritzen, J.C., Bartel, J., Becher, D., Gjermansen, M. et al. (2019) Identification and optimization of PrsA in *Bacillus subtilis* for improved yield of amylase. *Microb. Cell Fact.* **18**, 158–169, <https://doi.org/10.1186/s12934-019-1203-0>
- 30 Tjalsma, H., Noback, M.A., Bron, S., Venema, G., Yamane, K. and Van Dijk, J.M. (1997) *Bacillus subtilis* contains four closely related type I signal peptidases with overlapping substrate specificities. *J. Biol. Chem.* **272**, 25983–25992, <https://doi.org/10.1074/jbc.272.41.25983>
- 31 Collier, D.N. (1994) Expression of *Escherichia coli* SecB in *Bacillus subtilis* facilitates secretion of the SecB-dependent maltose-binding protein of *E. coli*. *J. Bacteriol.* **176**, 4937–4940, <https://doi.org/10.1128/JB.176.16.4937-4940.1994>
- 32 Kouwen, T.R., van der Goot, A., Dorenbos, R., Winter, T., Antelmann, H., Plaisier, M.C. et al. (2007) Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. *Mol. Microbiol.* **64**, 984–999, <https://doi.org/10.1111/j.1365-2958.2007.05707.x>
- 33 Kakeshita, H., Kageyama, Y., Ara, K., Ozaki, K. and Nakamura, K. (2010) Enhanced extracellular production of heterologous proteins in *Bacillus subtilis* by deleting the C-terminal region of the SecA secretory machinery. *Mol. Biotechnol.* **46**, 250–257, <https://doi.org/10.1007/s12033-010-9295-0>
- 34 Diao, L., Dong, Q., Xu, Z., Yang, S., Zhou, J. and Freudl, R. (2012) Functional implementation of the posttranslational SecB-SecA protein-targeting pathway in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **78**, 651–659, <https://doi.org/10.1128/AEM.07209-11>
- 35 Neef, J., Bongiorno, C., Goosens, V.J., Schmidt, B. and van Dijk, J.M. (2017) Intramembrane protease RasP boosts protein production in *Bacillus*. *Microb. Cell Fact.* **16**, 57–107, <https://doi.org/10.1186/s12934-017-0673-1>
- 36 Bolhuis, A., Tjalsma, H., Smith, H.E., de Jong, A., Meima, R., Venema, G. et al. (1999) Evaluation of bottlenecks in the late stages of protein secretion in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **65**, 2934–2941, <https://doi.org/10.1128/AEM.65.7.2934-2941.1999>
- 37 Neef, J., Bongiorno, C., Schmidt, B., Goosens, V.J. and van Dijk, J.M. (2020) Relative contributions of non-essential Sec pathway components and cell envelope-associated proteases to high-level enzyme secretion by *Bacillus subtilis*. *Microb. Cell Fact.* **19**, 52–102, <https://doi.org/10.1186/s12934-020-01315-2>
- 38 Aguilar Suarez, R., Stulke, J. and van Dijk, J.M. (2019) Less is more: towards a genome-reduced *Bacillus* cell factory for ‘difficult proteins’. *ACS Synth. Biol.* **8**, 99–108, <https://doi.org/10.1021/acssynbio.8b00342>
- 39 Reuss, D.R., Altenbuchner, J., Mader, U., Rath, H., Ischebeck, T., Sappa, P.K. et al. (2017) Large-scale reduction of the *Bacillus subtilis* genome: consequences for the transcriptional network, resource allocation, and metabolism. *Genome Res.* **27**, 289–299, <https://doi.org/10.1101/gr.215293.116>
- 40 Neef, J., Koedijk, D.G., Bosma, T., van Dijk, J.M. and Buist, G. (2014) Efficient production of secreted staphylococcal antigens in a non-lysing and proteolytically reduced *Lactococcus lactis* strain. *Appl. Microbiol. Biotechnol.* **98**, 10131–10141, <https://doi.org/10.1007/s00253-014-6030-y>
- 41 Neef, J., Milder, F.J., Koedijk, D.G., Klaassens, M., Heezius, E.C., van Strijp, J.A. et al. (2015) Versatile vector suite for the extracytoplasmic production and purification of heterologous His-tagged proteins in *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* **99**, 9037–9048, <https://doi.org/10.1007/s00253-015-6778-8>
- 42 Samazan, F., Rokbi, B., Seguin, D., Telles, F., Gautier, V., Richarme, G. et al. (2015) Production, secretion and purification of a correctly folded staphylococcal antigen in *Lactococcus lactis*. *Microb. Cell Fact.* **14**, 104–115, <https://doi.org/10.1186/s12934-015-0271-z>
- 43 Romero Pastrana, F., Neef, J., van Dijk, J.M. and Buist, G. (2017) A *Lactococcus lactis* expression vector set with multiple affinity tags to facilitate isolation and direct labeling of heterologous secreted proteins. *Appl. Microbiol. Biotechnol.* **101**, 8139–8149, <https://doi.org/10.1007/s00253-017-8524-x>
- 44 Kok, J., van Dijk, J.M., van der Vossen, J.M. and Venema, G. (1985) Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. *Appl. Environ. Microbiol.* **50**, 94–101, <https://doi.org/10.1128/AEM.50.1.94-101.1985>
- 45 Mierau, I., Kunji, E.R., Venema, G. and Kok, J. (1997) Casein and peptide degradation in lactic acid bacteria. *Biotechnol. Genet. Eng. Rev.* **14**, 279–301, <https://doi.org/10.1080/02648725.1997.10647945>
- 46 Cortes-Perez, N.G., Poquet, I., Oliveria, M.N., Gratadoux, J.J., Madsen, S.M., Miyoshi, A. et al. (2006) Construction and characterization of a *Lactococcus lactis* strain deficient in intracellular ClpP and extracellular HtrA proteases. *Microbiology* **152**, 2611–2618, <https://doi.org/10.1099/mic.0.28698-0>

- 47 Neef, J., Koedijk, D.G., Bosma, T., van Dijk, J.M. and Buist, G. (2014) Efficient production of secreted staphylococcal antigens in a non-lysing and proteolytically reduced *Lactococcus lactis* strain. *Appl. Microbiol. Biotechnol.* **98**, 10131–10141, <https://doi.org/10.1007/s00253-014-6030-y>
- 48 Miyoshi, A., Poquet, I., Azevedo, V., Commissaire, J., Bermudez-Humaran, L.G., Domakova, E. et al. (2002) Controlled production of stable heterologous proteins in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **68**, 3141–3146, <https://doi.org/10.1128/AEM.68.6.3141-3146.2002>
- 49 Sriraman, K. and Jayaraman, G. (2008) HtrA is essential for efficient secretion of recombinant proteins by *Lactococcus lactis*. *Appl. Environ. Microbiol.* **74**, 7442–7446, <https://doi.org/10.1128/AEM.00638-08>
- 50 Nouaille, S., Morello, E., Cortes-Perez, N.G., Le Loir, Y., Commissaire, J., Grataudoux, J.J. et al. (2006) Complementation of the *Lactococcus lactis* secretion machinery with *Bacillus subtilis* SecDF improves secretion of *Staphylococcal* Nuclease. *Appl. Environ. Microbiol.* **72**, 2272–2279, <https://doi.org/10.1128/AEM.72.3.2272-2279.2006>
- 51 Drouault, S., Anba, J., Bonneau, S., Bolotin, A., Ehrlich, S.D. and Renault, P. (2002) The peptidyl-prolyl isomerase motif is lacking in PmpA, the PrsA-like protein involved in the secretion machinery of *Lactococcus lactis*. *Appl. Environ. Microbiol.* **68**, 3932–3942, <https://doi.org/10.1128/AEM.68.8.3932-3942.2002>
- 52 Venema, R., Tjalsma, H., van Dijk, J.M., de Jong, A., Leenhouts, K., Buist, G. et al. (2003) Active lipoprotein precursors in the Gram-positive eubacterium *Lactococcus lactis*. *J. Biol. Chem.* **278**, 14739–14746, <https://doi.org/10.1074/jbc.M209857200>
- 53 Guillot, A., Boulay, M., Chambellon, E., Gitton, C., Monnet, V. and Juillard, V. (2016) Mass spectrometry analysis of the extracellular peptidome of *Lactococcus lactis*: lines of evidence for the coexistence of extracellular protein hydrolysis and intracellular peptide excretion. *J. Proteome Res.* **15**, 3214–3224, <https://doi.org/10.1021/acs.jproteome.6b00424>
- 54 Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijk, J.M. et al. (2001) A proteomic view on genome-based signal peptide predictions. *Genome Res.* **11**, 1484–1502, <https://doi.org/10.1101/gr.182801>
- 55 Bendtsen, J.D., Kiemer, L., Fausboll, A. and Brunak, S. (2005) Non-classical protein secretion in bacteria. *BMC Microbiol.* **5**, 58–2180, <https://doi.org/10.1186/1471-2180-5-58>
- 56 Ebner, P. and Gotz, F. (2019) Bacterial excretion of cytoplasmic proteins (ECP): occurrence, mechanism, and function. *Trends Microbiol.* **27**, 176–187, <https://doi.org/10.1016/j.tim.2018.10.006>
- 57 Krishnappa, L., Dreisbach, A., Otto, A., Goosens, V.J., Cranenburgh, R., Harwood, C.R. et al. (2013) Extracytoplasmic proteases determining the cleavage and release of secreted proteins, lipoproteins, and membrane proteins in *Bacillus subtilis*. *J. Proteome Res.* **12**, 4101–4110, <https://doi.org/10.1021/pr400433h>
- 58 Toyofuku, M., Carcamo-Oyarce, G., Yamamoto, T., Eisenstein, F., Hsiao, C.C., Kurosawa, M. et al. (2017) Prophage-triggered membrane vesicle formation through peptidoglycan damage in *Bacillus subtilis*. *Nat. Commun.* **8**, 481–017, <https://doi.org/10.1038/s41467-017-00492-w>
- 59 Ebner, P., Luqman, A., Reichert, S., Hauf, K., Popella, P., Forchhammer, K. et al. (2017) Non-classical protein excretion is boosted by PSMalpha-induced cell leakage. *Cell. Rep.* **20**, 1278–1286, <https://doi.org/10.1016/j.celrep.2017.07.045>
- 60 Ebner, P., Rinker, J. and Gotz, F. (2016) Excretion of cytoplasmic proteins in *Staphylococcus* is most likely not due to cell lysis. *Curr. Genet.* **62**, 19–23, <https://doi.org/10.1007/s00294-015-0504-z>
- 61 Pasztor, L., Ziebandt, A.K., Nega, M., Schlag, M., Haase, S., Franz-Wachtel, M. et al. (2010) Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. *J. Biol. Chem.* **285**, 36794–36803, <https://doi.org/10.1074/jbc.M110.167312>
- 62 Wang, G., Xia, Y., Gu, Z., Zhang, H., Chen, Y.Q., Chen, H. et al. (2015) A new potential secretion pathway for recombinant proteins in *Bacillus subtilis*. *Microb. Cell Fact.* **14**, 179, <https://doi.org/10.1186/s12934-015-0374-6>
- 63 Ebner, P., Rinker, J., Nguyen, M.T., Popella, P., Nega, M., Luqman, A. et al. (2016) Excreted cytoplasmic proteins contribute to pathogenicity in *Staphylococcus aureus*. *Infect. Immun.* **84**, 1672–1681, <https://doi.org/10.1128/IAI.00138-16>
- 64 Radisky, D.C., Stallings-Mann, M., Hirai, Y. and Bissell, M.J. (2009) Single proteins might have dual but related functions in intracellular and extracellular microenvironments. *Nat. Rev. Mol. Cell Biol.* **10**, 228–234, <https://doi.org/10.1038/nrm2633>
- 65 Huberts, D.H. and van der Klei, I.J. (2010) Moonlighting proteins: an intriguing mode of multitasking. *Biochim. Biophys. Acta* **1803**, 520–525, <https://doi.org/10.1016/j.bbamcr.2010.01.022>
- 66 Kainulainen, V. and Korhonen, T.K. (2014) Dancing to another tune-adhesive moonlighting proteins in bacteria. *Biology (Basel)* **3**, 178–204, <https://doi.org/10.3390/biology3010178>
- 67 Wang, G., Xia, Y., Cui, J., Gu, Z., Song, Y., Chen, Y.Q. et al. (2014) The roles of moonlighting proteins in bacteria. *Curr. Issues Mol. Biol.* **16**, 15–22
- 68 Henderson, B. and Martin, A. (2013) Bacterial moonlighting proteins and bacterial virulence. *Curr. Top. Microbiol. Immunol.* **358**, 155–213, https://doi.org/10.1007/82_2011_188
- 69 Chen, J., Zhao, L., Fu, G., Zhou, W., Sun, Y., Zheng, P. et al. (2016) A novel strategy for protein production using non-classical secretion pathway in *Bacillus subtilis*. *Microb. Cell Fact.* **15**, 69–016, <https://doi.org/10.1186/s12934-016-0469-8>
- 70 Yang, C.K., Ewis, H.E., Zhang, X., Lu, C.D., Hu, H.J., Pan, Y. et al. (2011) Nonclassical protein secretion by *Bacillus subtilis* in the stationary phase is not due to cell lysis. *J. Bacteriol.* **193**, 5607–5615, <https://doi.org/10.1128/JB.05897-11>
- 71 Zhao, L., Chen, J., Sun, J. and Zhang, D. (2017) Multimer recognition and secretion by the non-classical secretion pathway in *Bacillus subtilis*. *Sci. Rep.* **7**, 44023, <https://doi.org/10.1038/srep44023>