Essays in Biochemistry (2021) **65** 187–195
https://doi.org/10.1042/EBC20200171

PORTLAND PRESS

Review Article

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

Jolanda Neef, Jan Maarten van Dijl* and Girbe Buist*

Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, P.O. Box 30001, 9700 RB Groningen, The Netherlands

Correspondence: Jan Maarten van Dijl (j.m.van.dijl01@umcg.nl)

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 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

*These authors contributed equally to this work.

Received: 12 March 2021
Revised: 15 April 2021
Accepted: 16 April 2021

Version of Record published: 26 July 2021

Essays in Biochemistry (2021) **65** 187–195
https://doi.org/10.1042/EBC20200171

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-glyceryl

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 (Δ*aprE*, Δ*nprE*), WB600 (Δ*nprE*, Δ*aprE*, Δ*epr*, Δ*bpr*, Δ*mpr*, Δ*nprB*), WB700 (WB600 Δ*vpr*) and WB800 (WB700 Δ*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-α2b [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

Essays in Biochemistry (2021) **65** 187–195
https://doi.org/10.1042/EBC20200171

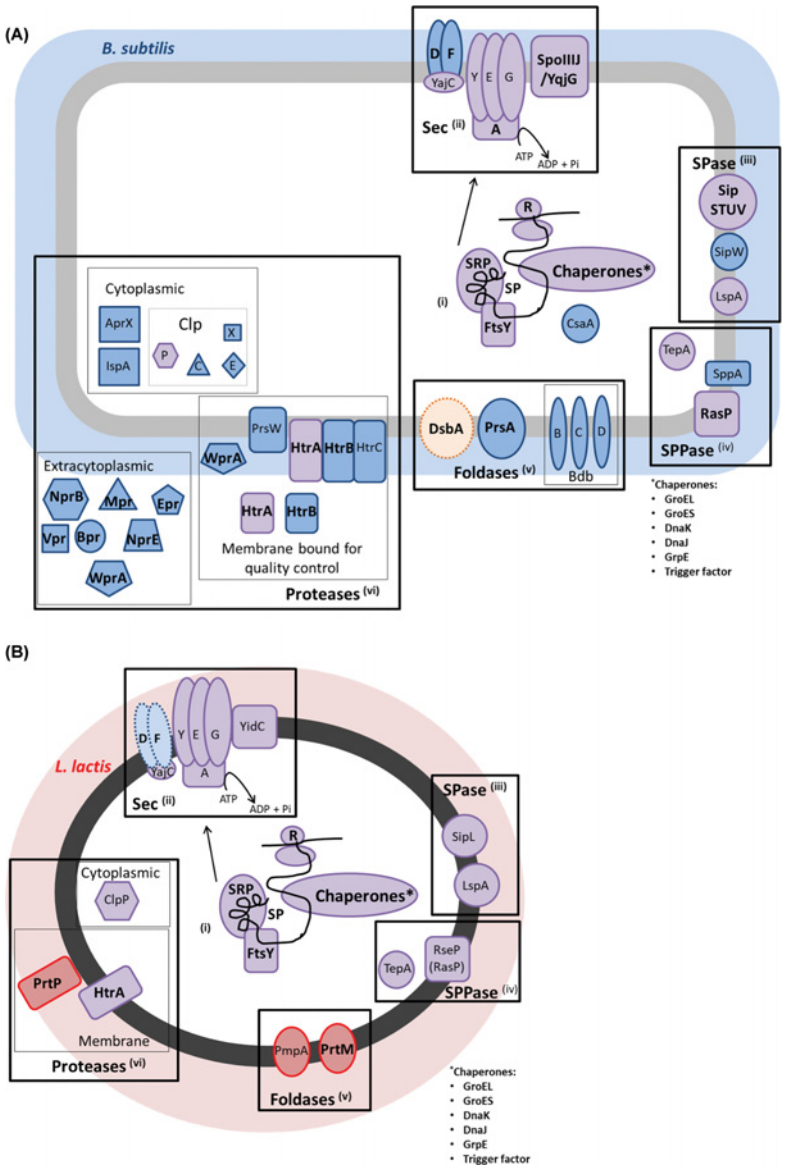

Figure 1. Comparison of the main components of the Sec-dependent protein translocation machinery of *B. subtilis* and *L. lactis*

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 SpoIIIJ/YqjG 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 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,

Essays in Biochemistry (2021) **65** 187–195
https://doi.org/10.1042/EBC20200171

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.

Funding
The authors are employed by the University Medical Center Groningen.

Open Access
Open access for this article was enabled by the participation of University of Groningen in an all-inclusive *Read & Publish* pilot with Portland Press and the Biochemical Society.

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 Dijl, 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 Dijl, 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 Dijl, 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 Dijl, 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
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 Dijl, 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 Dijl, 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

Essays in Biochemistry (2021) **65** 187–195
https://doi.org/10.1042/EBC20200171

21 Westers, L., Dijkstra, D.S., Westers, H., van Dijl, 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 Dijl, 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**, 92–015, 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**, 158–019, https://doi.org/10.1186/s12934-019-1203-0
30 Tjalsma, H., Noback, M.A., Bron, S., Venema, G., Yamane, K. and Van Dijl, 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., Bongiorni, C., Goosens, V.J., Schmidt, B. and van Dijl, J.M. (2017) Intramembrane protease RasP boosts protein production in *Bacillus*. *Microb. Cell Fact.* **16**, 57–017, 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., Bongiorni, C., Schmidt, B., Goosens, V.J. and van Dijl, 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**, 52–020, https://doi.org/10.1186/s12934-020-01315-2
38 Aguilar Suarez, R., Stulke, J. and van Dijl, 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 Dijl, 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**, 104–015, https://doi.org/10.1186/s12934-015-0271-z
43 Romero Pastrana, F., Neef, J., van Dijl, 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 Dijl, 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 Dijl, 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., Gratadoux, 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 Dijl, 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 Dijl, 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

Supplementary Table 1: Protein production and secretion in *B. subtilis* protease-deficient strains

Product (origin) / Naturally secreted via Sec (Y/N*)[1,2]	Producing vector/Promoter/SP[1]	Strain	Secreted yield	Biological active/ Degradation*	Ref.**
L-asparaginase (ASN) (*B. subtilis*) / Y (original SP mentioned[2])	pP43NMK/P43 variant (B2, −28:A→G, 13:A→G)/WapA	WB600	After deletion of the N-terminal 25-residues of ASN 407.6 U/mL (2.5 g/L)	Y/N	[1]
Pullulanase (*Bacillus naganoensis*) / N	pMA0911/P*hpaII* or P43/LipA	WB600, WB800	Best yield obtained using WB600 in combination with SP-*lipA* and P43 (24.5 ± 0.3 U/ml, 6.28x better than original (WB800 SP-*lipA* and P-*hpaII*)	Y/n.d.	[2]
Recombinant alkaline α-amylase (Unknown origin) / Y (mentioned in text[2])	pMA0911-*amy*/P*hpaII*/n.d.	WB600 mut-12#	Yield increased with 35.0% and productivity with 8.8%, the extracellular protein concentration increased 37.9% when compared to WB600	Y/N	[3]
CotA-laccase (*Bacillus pumilus*) / N	pMA0911/ P*hpaII*/WapA	WB600	373.1 U/mL	Y/n.d.	[4]
γ-Polyglutamic acid synthetase (*B. subtilis)* / N	pWB980/P43/n.d.	WB600	1.74 g/L	n.d./N	[5]
Cellobiohydrolases (*cbhA*, *celK*, *celO*, *cel48Y*, *cel48S*) and endoglucanase (*celA*) (*Clostridium thermocellum)* / n.d.	pP43JM2/P43/NprB	WB600, WB800	Efficient secretion for all but CelO. No quantification	Y/I	[6]
Keratinase (*Bacillus licheniformis*) / Y (JQ894491[2])	pMA0911/P*hpaII*/native	WB600	323 units/mL (non-induced)	Y/N	[7]
Xylanase (*Thermoanaerobacterium sp*) / Y (mentioned in text[2])	pJX33/P*bj31*/native	WB800	(8.4U/ml)	Y/Y	[8]

Bacillopeptidase F (*B. subtilis*) / Y (KT259045[2])	pMD18-T/native/+Proseq, native	WB700	n.d.	Y/Y	[9]
Synthesized cecropin A-melittin mutant (U) / n.d.	pDM030/P*glv*(maltose-inducible)/SacB	WB700	159 mg/L	Y/N	[10]
α-Amylase (*Bacillus amyloliquefaciens*) /Y (mentioned in text[2])	pP43X/P43/32aa (non-specified)	WB800	5566 U/mg, 1.48x increased production in WB800, when compared to wildtype	Y/I	[11]
Lysozyme (*Bacillus licheniformis*) / Y (mentioned in text)	pGJ203/P43 fused to P*sacB*/SacB	WB800	78 U/ml	Y/?	[12] ***
Protease (*Halobacillus* sp) / n.d.	pSaltExSePR5/P*opuAA* (NaCl induction)/SubE	WB800	9.1 U/ml	Y/I	[13]
β-1,3-1,4-glucanase (LicB) (*C. thermocellum*) / Y (mentioned in text[2])	pP43JM2/P43/NprB	WB800	1.18 U/g cell mass	Y/Y	[14]
Nattokinase (*B. subtilis* VTCC-DVN-12-01) / Y (EF061457[2])	n.d./P*acoA*/n.d.	WB800	600 mg/L	Y/N	[15]
Cellobiose 2-epimerase from (*Caldicellulosiruptor saccharolyticus*) / n.d.	pMA09/n.d./n.d.	WB800	5.3 U/mL	Y/Y	[16]
PA (component of the anthrax toxin)(*Bacillus anthracis*) / n.d.	pHT28*pagA*/P*grac*/AmyL	BRB07, BRB08, BRB11, BRB12, BRB14	BRB07: Approximately 1 g/L, BRB08: Slightly more than 1 g/L, BRB11-14: Slightly less than 1 g/L	n.d./Y in BRB07-12, hardly any degradation in BRB14	[17]
1,3-1,4-β-glucanase(U) / n.d.		WB600	2493.8 U/mL	Y/n.d.	[18]

Paramyosin (*Clororchis sinensis*) / N (cyst wall)	pEB03-CotC-CsPmy/PcotC/spore located	WB600	n.d.	Y/n.d.	[19]
Pullulanase (*Bacillus naganoensis*) / N	pMA0911/ Psac/LipA	WB600, WB800	Simultaneous DegQ production let to 60% increased enzyme activity in WB800. 26.5 U/ml	Y/n.d.	[20]
β-Mannanase *(Bacillus clausii*) / Y (WP_041823500.1[2])	Pma5/P43/LipA	WB600	1050 U/ml	Y/n.d.	[21]
Phospholipase D (*Streptomyces racemochromogenes*) / Y (AB573232[2])	pMA0911/HpaII/AmyE RBS optimized	WB600	24.2 U/ml	Y/n.d.	[22]
L-Asparaginase (*Bacillus cereus*) / N	pP43NMK/P43/AmyE	WB600	374.9 U/ml	Y/n.d.	[23]
GH30-8 endoxylanase (*B. subtilis*) / Y (mentioned in text[2])	pMA05/n.d./n.d.	WB800	55 U/mL	Y/N	[24]
Cel8A endoglucanase fused to LysM cell wall binding module (*C. thermocellum)* / n.d.	pBL113/P IPTG inducible/ PhrC Integration vector	WB800, BRB07, BRB08, BRB14	n.d. BRB08 highest production without causing secretion stress	Y/Y	[25]
Endo-inulinase (*Pseudomonas mucidolens*) / n.d.	PHY300PLK/P43/NprB Integration vector	WB800-R	67.84 ± 0.72 g/L	Y/n.d.	[26]
Nattokinase / Y (mentioned in text[2])	pBSG03/P08 (CodY biding sequence deleted)/WapA	WB800	292 FU/ml	Y/N	[27]
β-galactosidase (*Bacillus megaterium*) / n.d.	pMA05/n.d./n.d..	WB800	17.55 U/ml	Y/n.d.	[28]
Chitinase (*Bacillus* sp. DAU101) / Y (mentioned in text[2])	pP43NMK/P43/NprB also RBS optimized	WB600	51.67 U/mL	Y/reduced when compared to previous results	[29]

Pullulanase (*Bacillus naganoensis*) / N	Chromosomally inserted/PHpaII/LipA	WB800 WB600	30.32 U/ml 18.83 U/ml	Y/N Y/Y	[30]
Alkaline serine protease (BcaPRO, *Bacillus clausii*) / Y (FJ940727.1[2])	pWPROn/PBsamy-PBaamy/DacB	WB600	27,860 U/ml	Y/n.d.	[31]
Cyclomaltodextrin glucanotransferase (*Bacillus firmus*) / Y (KF270899)	pWB980/P43/SacB	WB800	1.33 µmol β-CD/min/mL	Y/Y	[32]
β-mannanase (*Bacillus* sp. MK-2) / Y (CAB12407.2[2])	pP43NMK/P43/NprB	WB800	2802 U/mg	Y/n.d.	[33]
Nattokinase (*B. subtilis* natto) / Y (mentioned in text[2])	pMA0911-*wapA*-*pro*-NK/PHpaII-PHpaII-PP43/WapA	WB800	816.7 ± 30.0 FU/mL	Y/N	[34]
CIPS/SCIN/IsaA/Nuc (*Stapylococcus aureus*) / Y (mentioned in text[2])	pRAG3/PspaS/AmyQ	PG10 (mini *Bacillus*)	Production in mini-*Bacillus* when this was not possible in the wt *Bacillus* strain (168)	Y/Y	[35]
Diacetylchitobiose deacetylase R157T (*Pyrococcus horikoshii*) / N	pMA0911/P43/ YncM	WB600	3,112.2 U/mg	Y/Y	[36]
α-amylase (AmySA K82E/S405R, *Bacillus stearothermophilus*) / Y (mentioned in text[2])	pBE/PaprE/YojL	WS11 (Δ*hrcA*)****	9201.1 U mL-1	Y/n.d.	[37]
Recombinant keratinase, KerZ1 (*Bacillus licheniformis*) / Y (mentioned in text[2])	pP43NMK/p43/NprB	WB600	426.60 KU/mL	Y/N	[38]
Polyethylene terephthalate hydrolase (PETase, *Ideonella sakaiensis*) / Y (mentioned in text[2])	pUBC19/P43/Amy	WB600	Increased 8 fold when compared to wt SP	Y/n.d.	[39]
Adenylate deaminase (AMPF, *Aspergillus oryzae* GX-08) / Y (mentioned in text[2])	pMA5/PHpaII/n.d..	WB600	2540 U/mL	Y/n.d.	[40]
Chlorothalonil hydrolytic dehalogenase (Chd, n.d.) / n.d.	Integrated in chromosome/PaprE	WB800	2622 U/L	Y/n.d.	[41]
OUC-Lipase 6 (*Streptomyces violascens*) / N	pP43NMK/P43/NprB	WB800	Increased activity 3.24 fold	Y/n.d.	[42]

Lantibiotics (*B. subtilis*) / Y (mentioned in text[2])	Integration in chromosome/Pspank-hy/leader peptide SpaS	PG10 (mini *Bacillus)*	n.d., clean background to simplify downstream processing	Y/n.d.	[43]

[1]Verified with SignalP4.1 if a Genbank accession was available
[2]Where appropriate the Genbank accession code or a reference to the main manuscript is indicated
[*]Y, yes; N, no; n.d., not described; I, inconclusive
[**]References cover the period between 2012 and 2020
[***]Only the abstract is publicly available.
[****]Mutation results in overexpression of chaperones GroEL-GroES and DnaK-DnaJ-GrpE

Supplementary Table 2: **Protein production and secretion in *L. lactis,* using various strains and expression systems**

Product / Naturally secreted via Sec (Y/N*)[1,2]	**Producing vector/Promoter/SP[1]**	**Strain**	**Secreted yield**	**Biologically active/ recognized***	**Ref****
Virulence factors (SA0620, FtsL, ClfB, SA2100, Pro-Atl, IsdB) (*Staphylococcus aureus*) / Y (mentioned in text[2])	pNG4110/P*nisA*/Usp45 pNG4111/P*nisA*/Usp45 pNG4210/P*nisA*/Usp45	PA1001	mg/L range, post-translational phosphorylation obtained	Y (partially done)/Y	[44]
Virulence factors (HtrA1, HtrA2)(*S. aureus*) / Y (KF322112 and KF322111[2])	pLB145/P*ZitR*/Exp4	MG1363	2.5 and 2.2 mg/L (HtrA1 and HtrA2 resp.) Max 7 mg	Y/N	[45]
S. aureus virulence factors (LytM, Nuc, Aly, SA0620, SA2097) / Y (mentioned in text[2])	pNG400/P*nisA*/Usp45	PA1001	22; 18.8; 1.4; 4.2 mg/L (LytM, Nuc, SA2097, Aly resp.)	Y/Y	[46]
β-Cyclodextrin Glucanotransferase / Y (mentioned in text[2])	pNZ8048/P*nisA*/native-SP (NSP), SPK1 or Usp45	NZ9000	SPK1 secretion efficiency higher than Usp45. Total protein production higher for Usp45-fusion proteins	Y/Y	[47]
Designed Ankyrin Repeat Proteins (DARPins) fused to LysM-domain / n.d.	pNZ8148/P*nisA*/Usp45	NZ9000	n.d.	Y/N	[48]
Murine IL-10, murine TGF-β1, human Elafin, murine SLP-1 / Y (mentioned in text[2])	pSEC/P*nisA*/Usp45	NZ9000 (wt) and NZ9000Δ*htrA* (Δ*htrA*)	IL-10: 40 ng/ml (wt) TGF-β1:50 ng/ml (wt) Elafin: 37 ng/ml (wt) *vs* 55 ng/ml (Δ*htrA*) SLP-1: 100% secreted, yield not quantified (wt)	Y/I	[49]
Kisspeptin / Y (BC022819.1[2])	pNZ401/P*nisA*/Usp45 + LEISSTCDA pro-peptide	NZ9000	27.9 µg/ml	Y/I	[50]
B. subtilis originated Subtilisin QK-2 / Y (AJ579472.2[2])	pRF01 (pNZ8149 derivative)/P*nisA*/Usp45	NZ9000 NZ3900	Nd	Y/I	[51]

Mouse heme oxygenase-1 / n.d.	pNZ8148#2:SEC/P*nisA*/Usp45	NZ9000	Intracellular production, 5.37 ug/ml	Y/N	[52]
Pancreatitis-associated protein I (PAP) / n.d.	pSEC/P*nisA*/Usp45	NZ9000	n.d.	Y/n.d.	[53]
Human interleukin 22 (IL-22) / Y (mentioned in text[2])	pSEC/P*nisA*/Usp45	NZ9000	10 ng/ml	Y/Y	[54]
HSP65-6IA2P2 as autoantigens against T1 diabetes / n.d.	pCYT/P*nisA* and pHJ/P32/Usp45	NZ9000	n.d.	Y/n.d.	[55]
Carcinoembryonic antigen / n.d.	pSEC:LEISS/P*nisA*/Usp45 + LEISSTCDA pro-peptide	NZ9000	Surface display on *L. lactis* cells using LcsB anchor	Y/Y	[56]
Infectious bronchitis virus (IBV) multi-epitope EpiC fused to protein A anchor *S. aureus* / n.d.	pNZ8149/P*nisA*/Usp45	NZ3900	27 mg/L	Y/Y	[57]
Campylobacter jejuni originated CjaA antigen presenting CjaD peptide epitopes fused to the C-terminus of the *L. lactis* YndF containing the LPTXG motif / Y (mentioned in text[2])	pUWM1000/P*usp45*/Usp45	IL1403	n.d., however protein was detected on the surface of the L. lactis IL1403 producing cells	Y/I	[58]
Bacteriocin Pediocin PA-1 / Y (mentioned in text)	pSEC/P*nisA*/Usp45 + LEISSTCDA or SD pro-peptide	NZ9000	~2-4 µmol pediocin PA-1 equivalents/g of dry weight bacteria.	Y/n.d.	[59]
Leuconostoc mesenteroides originated glucansucrase (DsrI)*** / Y (mentioned in text[2])	pMSP3535H3/P*nisA*/Usp45,	LM0230	380 mg/L (at pH 6.0, in optimized fermentation set-up, high copy number plasmid (45–85 copies per cell))	Y/I	[60]
Thymic stromal lymphopoietin / n.d.	pLB333/P*groESL*/Exp4	MG1363	Maximum of 2500 pg/ml/OD bacteria, SICE system	Y/n.d.	[61]
Mature sakacin A (SakA) and its cognate immunity protein (SakI), two chimeras	pNZ8048/P*nisA*/Usp45 pMG36c/P32/Usp45	NZ9000	3.2 and 4.9 ug/mg dry weight bacteria producing EntP/SakA or SakA, resp.	Y/N	[62]

mimicking the N-terminal end of mature enterocin P (EntP/SakA) and mature enterocin A (EntA/SakA) together with SakI / mentioned in text; SakA contains 18 aa leader peptide, SakI not secreted					
Panel of 31 recombinant proteins (*Plasmodium falciparum*) / mentioned in text; diverse sub-cellular locations[2]	pSS1/n.d./n.d	MG1363	n.d.	Y/N	[63]
Phytase (*Escherichia coli*) / n.d.	pFUM003/PlacA/Usp45	NZ9000	19 U/ml	Y/N	[64]
E6 oncoprotein (Human papillomavirus) / n.d.	pNZ8123/PnisA/Usp45	NZ9000	n.d.	Y/N	[65]
Tumor necrosis factor-related apoptosis-inducing ligand (synthetic human) / N (transmembrane)	pNZ8124/PnisA/Usp45	NZ9000	97.4 ng/ml	Y/Y	[66]
Plantaricin E (*Lactobacillus plantarum*) / Y (mentioned in text)	pNZ8148/PnisA/PlnA	NZ3900	n.d.	Y/n.d.	[67]
Apical membrane antigen 1 (*Eimeria tenella*) / N (transmembrane)	pTX8048/PnisA/Usp45	NZ9000	n.d.	Y/Y	[68]
Nuclease (*S. aureus*) / Y (mentioned in text)	pS (integrative)/P170/Usp45	NZ9000	n.d.	Y/N	[69]
Microbial transglutaminase (*Streptomyces mobaraensis*) / Y (DQ132977)	pNZ8048/Pp5 or PnisA/Usp45 or Usp45(K2A)	NZ9000	43.5 ± 0.4 mg/L	Y/N	[70]
MPB70 (*Mycobacterium bovis*) / Y (mentioned in text[2])	pNZ8048e/PnisA/ Usp45TM8	NZ9000	n.d.	Y/n.d.	[71]
VP1 (enterovirus 71) / n.d. (located on virion surface)	pMG36e/P32/Usp45	MG1363	n.d.	Y/n.d.	[72]

β-1,3-1,4-glucanase (*Bacillus* sp. SJ-10) / n.d.	pNG8149/PnisA/Usp45	NZ3900	n.d.	Y/N	[73]
cyclodextrin glucanotransferase/ Y (mentioned in text[2])	pNG8048/PnisA/G1	NZ9000	16.89 U/ml	Y/n.d.	[74]
Tumour necrosis factor-related apoptosis-inducing ligand (Human) / N (transmembrane protein)	pNG7021/PpepN/Usp45	MG1363	n.d.	Y/Y	[75]
E7 oncoprotein (optiE7; human papillomavirus type 16) / Y (mentioned in text[2])	pNZ8123/PnisA/Usp45	NZ9000	35.49 µg/mL	Y/N	[76]
IMP1 (*Eimeria tenella*) / N (membrane protein)	pTX8048/PnisA/Usp45	NZ9000	n.d.	Y/n.d.	[77]
Circumsporozoite protein (*Plasmodium falciparum*) / Y (mentioned in text[2]; sporozoite surface located)	pSS1/n.d./n.d.	MG1363	25 mg/L	Y/n.d.	[78]

[1]Verified with SignalP4.1 if a Genbank accession was available
[2]Where appropriate the Genbank accession code or a reference to the main manuscript is indicated
[*]Y, yes; N, no; n.d., not described; I, inconclusive
[**]References cover the period between 2012 and 2020
[***]First publication describing industrial application of *L. lactis* for secreted protein production

REFERENCES

[1] Feng, Y., Liu, S., Jiao, Y., Gao, H., Wang, M., Du, G. et al. (2017) Enhanced extracellular production of L-asparaginase from *Bacillus subtilis* 168 by *B. subtilis* WB600 through a combined strategy. *Appl.Microbiol.Biotechnol.* **101**: 1509-1520, https://doi.org/10.1007/s00253-016-7816-x

[2] Song, W., Nie, Y., Mu, X.Q., Xu, Y. (2016) Enhancement of extracellular expression of *Bacillus naganoensis pullulanase* from recombinant *Bacillus subtilis*: Effects of promoter and host. *Protein Expr.Purif.* **124**: 23-31, https://doi.org/10.1016/j.pep.2016.04.008

[3] Ma, Y., Yang, H., Chen, X., Sun, B., Du, G., Zhou, Z., et al. (2015) Significantly improving the yield of recombinant proteins in *Bacillus subtilis* by a novel powerful mutagenesis tool (ARTP): Alkaline alpha-amylase as a case study. *Protein Expr.Purif.* **114**: 82-88, https://doi.org/10.1016/j.pep.2015.06.016

[4] Guan, Z.B., Shui, Y., Song, C.M., Zhang, N., Cai, Y.J., Liao, X.R. (2015) Efficient secretory production of CotA-laccase and its application in the decolorization and detoxification of industrial textile wastewater**.** *Environ.Sci.Pollut.Res.Int.* **22**: 9515-9523, https://doi.org/10.1007/s11356-015-4426-6

[5] Lin, B., Li, Z., Zhang, H., Wu, J., Luo, M. (2016) Cloning and Expression of the gamma-Polyglutamic Acid Synthetase Gene *pgsBCA* in *Bacillus subtilis* WB600. *Biomed.Res.Int.* **2016**: 3073949, https://doi.org/10.1155/2016/3073949

[6] Liu, J.M., Xin, X.J., Li, C.X., Xu, J.H., Bao, J. (2012) Cloning of thermostable cellulase genes of *Clostridium thermocellum* and their secretive expression in *Bacillus subtilis*. *Appl.Biochem.Biotechnol.* **166**: 652-662, https://doi.org/10.1007/s12010-011-9456-z

[7] Liu, B., Zhang, J., Li, B., Liao, X., Du, G., Chen, J. (2013) Expression and characterization of extreme alkaline, oxidation-resistant keratinase from *Bacillus licheniformis* in recombinant *Bacillus subtilis* WB600 expression system and its application in wool fiber processing. *World J.Microbiol.Biotechnol.* **29**: 825-832, https://doi.org/10.1007/s11274-012-1237-5

[8] Phuong, N.D., Jeong, Y.S., Selvaraj, T., Kim, S.K., Kim, Y.H., Jung, K.H., et al. (2012) Production of XynX, a large multimodular protein of *Thermoanaerobacterium* sp., by protease-deficient *Bacillus subtilis* strains [corrected]. *Appl.Biochem.Biotechnol.* **168**: 375-382, https://doi.org/10.1007/s12010-012-9781-x

[9] Meng, D., Dai, M., Xu, B.L., Zhao, Z.S., Liang, X., Wang, M., et al. (2015) Maturation of Fibrinolytic Bacillopeptidase F Involves both Hetero- and Autocatalytic Processes. *Appl.Environ.Microbiol.* **82**: 318-327, https://doi.org/10.1128/AEM.02673-15.

[10] Ji, S., Li, W., Baloch, A.R., Wang, M., Li, H., Cao, B., et al. (2017) Efficient biosynthesis of a Cecropin A-melittin mutant in *Bacillus subtilis* WB700. *Sci.Rep.* **7**: 40587, https://doi.org/10.1038/srep40587

[11] Chen, J., Chen, X., Dai, J., Xie, G., Yan, L., et al. (2015) Cloning, enhanced expression and characterization of an alpha-amylase gene from a wild strain in *B. subtilis* WB800. *Int.J.Biol.Macromol.* **80**: 200-207, https://doi.org/10.1016/j.ijbiomac.2015.06.018

[12] Zhang, H., Fu, G., Zhang, D. (2014) Cloning, characterization, and production of a novel lysozyme by different expression hosts. *J.Microbiol.Biotechnol.* **24**: 1405-1412, https://doi.org/10.4014/jmb.1404.04039.

[13] Promchai, R., Promdonkoy, B., Tanapongpipat, S., Visessanguan, W., Eurwilaichitr, L., Luxananil, P. (2016) A novel salt-inducible vector for efficient expression and secretion of heterologous proteins in *Bacillus subtilis*. *J.Biotechnol.* **222**: 86-93, https://doi.org/10.1016/j.jbiotec.2016.02.019

[14] Luo, Z., Gao, Q., Li, X., 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

[15] Nguyen, T.T., Quyen, T.D., Le, H.T. (2013) Cloning and enhancing production of a detergent- and organic-solvent-resistant nattokinase from *Bacillus subtilis* VTCC-DVN-12-01 by using an eight-protease-gene-deficient *Bacillus subtilis* WB800. *Microb.Cell.Fact.* **12**: 79-2859, https://doi.org/10.1186/1475-2859-12-79

[16] Wu, L., Xu, C., Li, S., Liang, J., Xu, H., Xu, Z. (2017) Efficient production of lactulose from whey powder by cellobiose 2-epimerase in an enzymatic membrane reactor. *Bioresour.Technol.* **233**: 305-312, https://doi.org/10.1016/j.biortech.2017.02.089

[17] 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

[18] Niu, C., Liu, C., Li, Y., Zheng, F., Wang, J., Li, Q. (2018) Production of a thermostable 1,3-1,4-beta-glucanase mutant in *Bacillus subtilis* WB600 at a high fermentation capacity and its potential application in the brewing industry. *Int.J.Biol.Macromol.* **107**: 28-34, https://doi.org/10.1016/j.ijbiomac.2017.08.139.

[19] Sun, H., Lin, Z., Zhao, L., Chen, T., Shang, M., Jiang, H. et al. (2018) *Bacillus subtilis* spore with surface display of paramyosin from *Clonorchis sinensis* potentializes a promising oral vaccine candidate. *Parasit.Vectors* 2018, **11**: 156-018, https://doi.org/10.1186/s13071-018-2757-0.

[20] Deng, Y., Nie, Y., Zhang, Y., Wang, Y., Xu, Y. (2018) Improved inducible expression of *Bacillus naganoensis* pullulanase from recombinant *Bacillus subtilis* by enhancer regulation. *Protein Expr.Purif.* **148**: 9-15, https://doi.org/10.1016/j.pep.2018.03.012

[21] Zhou, C., Xue, Y., Ma, Y. (2018) Characterization and high-efficiency secreted expression in *Bacillus subtilis* of a thermo-alkaline beta-mannanase from an alkaliphilic *Bacillus clausii* strain S10. *Microb.Cell.Fact.* **17**: 124-018, https://doi.org/10.1186/s12934-018-0973-0

[22] Huang, T., Lv, X., Li, J., Shin, H.D., Du, G., Liu, L. (2018) Combinatorial Fine-Tuning of Phospholipase D Expression by *Bacillus subtilis* WB600 for the Production of Phosphatidylserine. *J.Microbiol.Biotechnol.* **28**: 2046-2056, https://doi.org/10.4014/jmb.1806.06048

[23] Feng, Y., Liu, S., Jiao, Y., Wang, Y., Wang, M., Du, G. (2019) Gene cloning and expression of the l-asparaginase from *Bacillus cereus* BDRD-ST26 in *Bacillus subtilis* WB600. *J.Biosci.Bioeng.* **127**: 418-424, https://doi.org/10.1016/j.jbiosc.2018.09.007

[24] Guo, Y., Gao, Z., Xu, J., Chang, S., Wu, B., He, B. (2018) A family 30 glucurono-xylanase from *Bacillus subtilis* LC9: Expression, characterization and its application in Chinese bread making. *Int.J.Biol.Macromol.* **117**: 377-384, https://doi.org/10.1016/j.ijbiomac.2018.05.143

[25] Huang, G.L., Gosschalk, J.E., Kim, Y.S., Ogorzalek Loo, R.R., Clubb, R.T. (2018) Stabilizing displayed proteins on vegetative *Bacillus subtilis* cells. *Appl.Microbiol.Biotechnol.* **102**: 6547-6565, https://doi.org/10.1007/s00253-018-9062-x

[26] Jiang, R., Qiu, Y., Huang, W., Zhang, L., Xue, F., Ni, H., et al. (2018) One-Step Bioprocess of Inulin to Product Inulo-Oligosaccharides Using *Bacillus subtilis* Secreting an Extracellular Endo-Inulinase. *Appl.Biochem.Biotechnol.* **187**:116-128, https://doi.org/10.1007/s12010-018-2806-3

[27] Cui, W., Suo, F., Cheng, J., Han, L., Hao, W., Guo, J., et al. (2018) Stepwise modifications of genetic parts reinforce the secretory production of nattokinase in *Bacillus subtilis*. *Microb.Biotechnol.* **11**: 930-942, https://doi.org/10.1111/1751-7915.13298

[28] Zhao, L., Zhou, Y., Qin, S., Qin, P., Chu, J., He, B. (2018) beta-Galactosidase BMG without galactose and glucose inhibition: Secretory expression in *Bacillus subtilis* and for synthesis of oligosaccharide. *Int.J.Biol.Macromol.* **120**: 274-278, https://doi.org/10.1016/j.ijbiomac.2018.07.148

[29] Pan, M., Li, J., Lv, X., Du, G., Liu, L. (2019) Molecular engineering of chitinase from Bacillus sp. DAU101 for enzymatic production of chitooligosaccharides. *Enzyme Microb.Technol.* **124**: 54-62, https://doi.org/10.1016/j.enzmictec.2019.01.012

[30] Wang, Y., Chen, S., Zhao, X., Zhang, Y., Wang, X., Nie, Y., et al. (2019) Enhancement of the production of *Bacillus naganoensis* pullulanase in recombinant *Bacillus subtilis* by integrative expression. *Protein Expr.Purif.* **159**: 42-48, https://doi.org/10.1016/j.pep.2019.03.006

[31] Liu, Y., Shi, C., Li, D., Chen, X., Li, J., Zhang, Y., et al. (2019) Engineering a highly efficient expression system to produce BcaPRO protease in *Bacillus subtilis* by an optimized promoter and signal peptide. *Int.J.Biol.Macromol.* **138**: 903-911, https://doi.org/10.1016/j.ijbiomac.2019.07.175

[32] Gimenez, G.G., Costa, H., de Lima Neto, Q.A., Fernandez, M.A., Ferrarotti, S.A., Matioli, G. (2019) Sequencing, cloning, and heterologous expression of cyclomaltodextrin glucanotransferase of *Bacillus firmus* strain 37 in *Bacillus subtilis* WB800. *Bioprocess Biosyst Eng.* **42**: 621-629, https://doi.org/10.1007/s00449-018-02068-4

[33] Zhang, W., Liu, Z., Zhou, S., Mou, H., Zhang, R. (2019) Cloning and expression of a beta-mannanase gene from *Bacillus* sp. MK-2 and its directed evolution by random mutagenesis. *Enzyme Microb.Technol.* **124**: 70-78, https://doi.org/10.1016/j.enzmictec.2019.02.003

[34] Liu, Z., Zheng, W., Ge, C., Cui, W., Zhou, L., Zhou, Z. (2019) High-level extracellular production of recombinant nattokinase in Bacillus subtilis WB800 by multiple tandem promoters. *BMC Microbiol.* **19**: 89-019, https://doi.org/10.1186/s12866-019-1461-3

[35] Aguilar Suarez, R., Stulke, J., van Dijl, 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

[36] Jiang, Z., Niu, T., Lv, X., Liu, Y., Li, J., Lu, W., et al. (2019) Secretory Expression Fine-Tuning and Directed Evolution of Diacetylchitobiose Deacetylase by *Bacillus subtilis*. *Appl.Environ.Microbiol.* 2019, **85**: e01076-19, https://doi.org/10.1128/AEM.01076-19

[37] Yao, D., Su, L., Li, N., Wu, J. (2019) Enhanced extracellular expression of *Bacillus stearothermophilus* alpha-amylase in *Bacillus subtilis* through signal peptide optimization, chaperone overexpression and alpha-amylase mutant selection. *Microb.Cell.Fact.* **18**: 69-019, https://doi.org/10.1186/s12934-019-1119-8

[38] Peng, Z., Mao, X., Zhang, J., Du, G., Chen, J. (2020) Biotransformation of keratin waste to amino acids and active peptides based on cell-free catalysis. *Biotechnol.Biofuels* **13**: 61-020, https://doi.org/10.1186/s13068-020-01700-4

[39] Wang, N., Guan, F., Lv, X., Han, D., Zhang, Y., Wu, N., et al. (2020) Enhancing secretion of polyethylene terephthalate hydrolase PETase in *Bacillus subtilis* WB600 mediated by the SPamy signal peptide. *Lett.Appl.Microbiol.* **71**: 235-241, https://doi.org/10.1007/s00449-020-02288-7

[40] Ke, C., Li, S., Cui, Y., Qiu, H., Pang, Z. (2020) Effects of different expression systems on characterization of adenylate deaminase from *Aspergillus oryzae*. *Bioprocess Biosyst Eng.* **43**: 919-926, https://doi.org/10.1007/s00449-020-02288-7.

[41] Ye, B., Luo, Y., Zhong, B., Zhu, J., Huang, J., Gu, J., et al. (2020) High-level stable expression of gene for preparation of chlorothalonil hydrolytic dehalogenase and its application in elimination of chlorothalonil inhibition on bioconversion of lignocellulosic biomass. *J.Biosci.Bioeng.* **130**: 630-636, https://doi.org/10.1016/j.jbiosc.2020.08.003

[42] Gao, K., Chu, W., Sun, J., Mao, X. (2020) Identification of an alkaline lipase capable of better enrichment of EPA than DHA due to fatty acids selectivity and regioselectivity. *Food Chem.* **330**: 127225, https://doi.org/10.1016/j.foodchem.2020.127225

[43] van Tilburg, A.Y., van Heel, A.J., Stulke, J., de Kok, N.A.W., Rueff, A.S., Kuipers, O.P. (2020) Mini*Bacillus* PG10 as a Convenient and Effective Production Host for Lantibiotics. *ACS Synth.Biol.* **9**: 1833-1842, https://doi.org/10.1021/acssynbio.0c00194

[44] 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:** 9037-48, https://doi.org/10.1007/s00253-015-6778-8

[45] 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**: 104-015, https://doi.org/10.1186/s12934-015-0271-z

[46] Neef, J., Koedijk, D.G., Bosma, T., van Dijl, J.M., Buist, G. (2014) Efficient production of secreted staphylococcal antigens in a non-lysing and proteolytically reduced *Lactococcus lactis* strain. *Appl.Microbiol.Biotechnol.* **98:** 10131-41, https://doi.org/10.1007/s00253-014-6030-y

[47] Subramaniam, M., Baradaran, A., Rosli, M.I., Rosfarizan, M., Khatijah, Y., Raha, A.R. (2012) Effect of signal peptides on the secretion of beta-cyclodextrin glucanotransferase in *Lactococcus lactis* NZ9000. *J.Mol.Microbiol.Biotechnol.* **22**: 361-372, https://doi.org/10.1159/000343921

[48] Zadravec, P., Strukelj, B., Berlec, A. (2015) Improvement of LysM-mediated surface display of designed ankyrin repeat proteins (DARPins) in recombinant and nonrecombinant strains of *Lactococcus lactis* and *Lactobacillus* Species. *Appl.Environ.Microbiol.* **81**: 2098-2106, https://doi.org/10.1128/AEM.03694-14

[49] Bermudez-Humaran, L.G., Motta, J.P., Aubry, C., Kharrat, P., Rous-Martin, L., Sallenave J.M., et al. (2015) Serine protease inhibitors protect better than IL-10 and TGF-beta anti-inflammatory cytokines against mouse colitis when delivered by recombinant lactococci. *Microb.Cell.Fact.* **14**: 26-015, https://doi.org/10.1186/s12934-015-0198-4

[50] Zhang, B., Li, A., Zuo, F., Yu, R., Zeng, Z., Ma, H., et al. (2016) Recombinant *Lactococcus lactis* NZ9000 secretes a bioactive kisspeptin that inhibits proliferation and migration of human colon carcinoma HT-29 cells. *Microb.Cell.Fact.* **15**: 102-016, https://doi.org/10.1186/s12934-016-0506-7

[51] Mao, R., Zhou, K., Han, Z., Wang, Y. (2016) Subtilisin QK-2: secretory expression in Lactococcus lactis and surface display onto gram-positive enhancer matrix (GEM) particles. *Microb.Cell.Fact.* **15**: 80-016, https://doi.org/10.1186/s12934-016-0478-7

[52] Shigemori, S., Watanabe, T., Kudoh, K., Ihara, M., Nigar, S., Yamamoto, Y., et al. (2015) Oral delivery of *Lactococcus lactis* that secretes bioactive heme oxygenase-1 alleviates development of acute colitis in mice. *Microb.Cell.Fact.* **14**: 189-015, https://doi.org/10.1186/s12934-015-0378-2

[53] Carvalho, R.D., Breyner, N., Menezes-Garcia, Z., Rodrigues, N.M., Lemos, L., Maioli, T.U., et al. (2017) Secretion of biologically active pancreatitis-associated protein I (PAP) by genetically modified dairy *Lactococcus lactis* NZ9000 in the prevention of intestinal mucositis. *Microb.Cell.Fact.* **16**: 27-017, https://doi.org/10.1186/s12934-017-0624-x

[54] Loera-Arias, M.J., Villatoro-Hernandez, J., Parga-Castillo, M.A., Salcido-Montenegro, A., Barboza-Quintana, O., Munoz-Maldonado, G.E., et al. (2014) Secretion of biologically active human interleukin 22 (IL-22) by *Lactococcus lactis*. *Biotechnol.Lett.* **36**: 2489-2494, https://doi.org/10.1007/s10529-014-1626-y

[55] Liu, K.F., Liu, X.R., Li, G.L., Lu, S.P., Jin, L., Wu, J. (2016) Oral administration of *Lactococcus lactis*-expressing heat shock protein 65 and tandemly repeated IA2P2 prevents type 1 diabetes in NOD mice. *Immunol.Lett.* **174**: 28-36, https://doi.org/10.1016/j.imlet.2016.04.008

[56] Zhang, X., Hu, S., Du, X., Li, T., Han, L., Kong, J. (2016) Heterologous expression of carcinoembryonic antigen in *Lactococcus lactis* via LcsB-mediated surface displaying system for oral vaccine development. *J.Microbiol.Immunol.Infect.* **49**: 851-858.

[57] Cao, H.P., Wang, H.N., Yang, X., Zhang, A.Y., Li, X., Ding, M.D., et al. (2013) *Lactococcus lactis* anchoring avian infectious bronchitis virus multi-epitope peptide EpiC induced specific immune responses in chickens. *Biosci.Biotechnol.Biochem.* **77**: 1499-1504, https://doi.org/10.1271/bbb.130157

[58] Kobierecka, P.A., Olech, B., Ksiazek, M., Derlatka, K., Adamska, I., Majewski, P.M., et al. (2016) Cell Wall Anchoring of the Campylobacter Antigens to *Lactococcus lactis*. *Front.Microbiol.* **7**: 165, https://doi.org/10.3389/fmicb.2016.00165

[59] Back, A., Borges, F., Mangavel, C., Paris, C., Rondags, E., Kapel, R., et al. (2016) Recombinant pediocin in *Lactococcus lactis*: increased production by propeptide fusion and improved potency by co-production with PedC. *Microb.Biotechnol.* **9**: 466-477, https://doi.org/10.1111/1751-7915.12285

[60] Skory, C.D., Cote, G.L. (2015) Secreted expression of *Leuconostoc mesenteroides* glucansucrase in *Lactococcus lactis* for the production of insoluble glucans. *Appl.Microbiol.Biotechnol.* 2015, **99**: 10001-10010, https://doi.org/10.1007/s00253-015-6854-0

[61] Aubry, C., Michon, C., Chain, F., Chvatchenko, Y., Goffin, L., Zimmerli, S.C., et al. (2015) Protective effect of TSLP delivered at the gut mucosa level by recombinant lactic acid bacteria in DSS-induced colitis mouse model. *Microb.Cell.Fact.* **14**: 176-015, https://doi.org/10.1186/s12934-015-0367-5

[62] Jimenez, J.J., Borrero, J., Diep, D.B., Gutiez, L., Nes, I.F., Herranz, C., et al. (2013) Cloning, production, and functional expression of the bacteriocin sakacin A (SakA) and two SakA-derived chimeras in lactic acid bacteria (LAB) and the yeasts Pichia pastoris and *Kluyveromyces lactis*. *J.Ind.Microbiol.Biotechnol.* **40**: 977-993, https://doi.org/10.1007/s10295-013-1302-6

[63] Singh, S.K., Tiendrebeogo, R.W., Chourasia, B.K., Kana, I.H., Singh, S., Theisen, M. (2018) *Lactococcus lactis* provides an efficient platform for production of disulfide-rich recombinant proteins from *Plasmodium falciparum*. *Microb.Cell.Fact.* **17**: 55-018, https://doi.org/10.1186/s12934-018-0902-2

[64] Pakbaten, B., Majidzadeh Heravi, R., Kermanshahi, H., Sekhavati, M.H., Javadmanesh, A., Mohammadi Ziarat, M., et al. (2019) Production of Phytase Enzyme by a Bioengineered Probiotic for Degrading of Phytate Phosphorus in the Digestive Tract of Poultry**.** *Probiotics Antimicrob.Proteins* **11:** 580-587, https://doi.org/10.1007/s12602-018-9423-x

[65] Taghinezhad, S.S., Mohseni, A.H., Keyvani, H., Razavilar, V. (2018) Protection against human papillomavirus type 16-induced tumors in C57BL/6 mice by mucosal vaccination with *Lactococcus lactis* NZ9000 expressing E6 oncoprotein. *Microb.Pathog.* **126**: 149-156, https://doi.org/10.1016/j.micpath.2018.10.043

[66] Ciacma, K., Wieckiewicz, J., Kedracka-Krok, S., Kurtyka, M., Stec, M., Siedlar, M., et al. (2018) Secretion of tumoricidal human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by recombinant *Lactococcus lactis*: optimization of in vitro synthesis conditions. *Microb.Cell.Fact.* **17**: 177-018, https://doi.org/10.1186/s12934-018-1028-2

[67] Mustopa, A.Z., Mariyah, S., Fatimah, Budiarti, S., Murtiyaningsih, H., Alfisyahrin, W.N. (2018) Construction, heterologous expression, partial purification, and in vitro cytotoxicity of the recombinant plantaricin E produced by *Lactococcus lactis* against Enteropathogenic *Escherichia coli* K.1.1 and human cervical carcinoma (HeLa) cells. *Mol.Biol.Rep.* **45**: 1235-1244, https://doi.org/10.1007/s11033-018-4277-6

[68] Li, J., Wang, F., Ma, C., Huang, Y., Wang, D., Ma, D. (2018) Recombinant *Lactococcus lactis* expressing *Eimeria tenella* AMA1 protein and its immunological effects against homologous challenge. *Exp.Parasitol.* **191**: 1-8, https://doi.org/10.1016/j.exppara.2018.05.003

[69] Koko, I., Song, A.A., Masarudin, M.J., Abdul Rahim, R. (2019) Engineering integrative vectors based on phage site-specific recombination mechanism for *Lactococcus lactis*. *BMC Biotechnol.* **19**: 82-019, https://doi.org/10.1186/s12896-019-0575-x

[70] Ma, T., Lu, J., Zhu, J., Li, X., Gu, H., Montalban-Lopez, M., et al. (2019) The Secretion of *Streptomyces monbaraensis* Transglutaminase From *Lactococcus lactis* and Immobilization on Porous Magnetic Nanoparticles. *Front.Microbiol.* **10**: 1675, https://doi.org/10.3389/fmicb.2019.01675

[71] Stedman, A., Chambers, M.A., Gutierrez-Merino, J. (2019) Secretion and functional expression of Mycobacterium bovis antigens MPB70 and MPB83 in lactic acid bacteria**.** *Tuberculosis* **117**: 24-30, https://doi.org/10.1016/j.tube.2019.05.007

[72] Xu, P., Wang, Y., Tao, L., Wu, X., Wu, W. (2019) Recombinant *Lactococcus lactis* secreting viral protein 1 of enterovirus 71 and its immunogenicity in mice. *Biotechnol.Lett.* 2019, **41**: 867-872, https://doi.org/10.1007/s10529-019-02695-1

[73] Tak, J.Y., Jang, W.J., Lee, J.M., Suraiya, S., Kong, I.S. (2019) Expression in *Lactococcus lactis* of a beta-1,3-1,4-glucanase gene from *Bacillus* sp. SJ-10 isolated from fermented fish. *Protein Expr.Purif.* **162**: 18-23, https://doi.org/10.1016/j.pep.2019.05.006

[74] Mahmud, H., Ismail, A., Abdul Rahim, R., Low, K.O., Md Illias, R. (2019) Enhanced secretion of cyclodextrin glucanotransferase (CGTase) by *Lactococcus lactis* using heterologous signal peptides and optimization of cultivation conditions. *J.Biotechnol.* 2019, **296**: 22-31, https://doi.org/10.1016/j.jbiotec.2019.02.013

[75] Bohlul, E., Hasanlou, F., Taromchi, A.H., Nadri, S. (2019) TRAIL-expressing recombinant *Lactococcus lactis* induces apoptosis in human colon adenocarcinoma SW480 and HCT116 cells. *J.Appl.Microbiol.* **126**: 1558-1567, https://doi.org/10.1111/jam.14237

[76] Mohseni, A.H., Taghinezhad-S, S., Keyvani, H., Razavilar, V. (2019) Extracellular overproduction of E7 oncoprotein of Iranian human papillomavirus type 16 by genetically engineered *Lactococcus lactis*. *BMC Biotechnol.* **19**: 8-019, https://doi.org/10.1186/s12896-019-0499-5

[77] Ma, C., Li, G., Chen, W., Jia, Z., Yang, X., Pan, X., et al. (2021) *Eimeria tenella*: IMP1 protein delivered by *Lactococcus lactis* induces immune responses against homologous challenge in chickens. *Vet.Parasitol.* 2021, **289**: 109320, https://doi.org/10.1016/j.vetpar.2020.109320

[78] Singh, S.K., Plieskatt, J., Chourasia, B.K., Singh, V., Bolscher, J.M., Dechering, K.J., et al. (2020) The *Plasmodium falciparum* circumsporozoite protein produced in *Lactococcus lactis* is pure and stable. *J.Biol.Chem.* **295**: 403-414, https://doi.org/10.1074/jbc.RA119.011268