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REVIEW PAPER

Engineering *Bacillus subtilis* Cells as Factories: Enzyme Secretion and Value-added Chemical Production

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Abstract *Bacillus subtilis* has been studied for more than half a century, ever since the dawn of molecular biology, as a representative Gram-positive bacterium and cell factory. Two characteristic capacities of *B. subtilis*, namely its natural competence for DNA uptake and high-level enzyme secretion, have been investigated and exploited intensively during these long years. As a consequence, this bacterium has evolved into an excellent platform for synthetic biological research and development. In this review, we outline basic concepts for *B. subtilis* cell factory engineering, and we describe several examples of its applications in the production of proteins and high-value metabolites. In particular, we highlight engineering approaches that can make the already very efficient *Bacillus* protein secretion pathways even more efficient for the production of enzymes and pharmaceutical proteins. We further showcase examples of metabolic engineering in *B. subtilis* based on synthetic biology principles to produce various high-value or health-promoting substances, especially inositol stereoisomers. We conclude that the versatile traits of *B. subtilis*, combined with multi-omics approaches and rapidly developing technologies for genome engineering and high-throughput screening enable us to modify and optimize this bacterium's metabolic circuits to deliver compounds that are needed for a green and sustainable society as well as a healthy population.

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1. Introduction

Bacillus subtilis is a well-known representative of the Gram-positive bacteria, and it has been used for basic and applied research for more than half a century, ever since the dawn of molecular biology [1-3]. The ample possibilities for genetic engineering of *B. subtilis* have been driving the technological development. *B. subtilis* is known to be particularly amenable to genetic manipulation, because of its efficient natural competence for DNA uptake [4]. It is capable of taking up not only chromosomal and plasmid DNA, but also PCR fragments that are synthesized *in vitro*. Thus, *B. subtilis* can acquire a wide range of genetic elements to implement desired and designed cellular functions, mechanisms and pathways. This is particularly attractive for studying artificially introduced functionalities in *B. subtilis* allowing the manipulation of transcription, translation, and metabolism [5-9].

Another virtue of *B. subtilis* is its prominent capacity to secrete large amounts of industrially relevant enzymes, including proteases, amylases, and lipases. Research aimed at improving the capacity of *B. subtilis* for the secretion of a variety of native and homologous proteins has been carried out over more than three decades. In particular, the expression of genes for secreted enzymes has been dramatically enhanced, and genes for extracellular proteases that can degrade the secreted enzymes have been deleted [10]. *B. subtilis* has evolved at least two fundamentally different pathways for protein export from their site of synthesis, the cytoplasm, to the extracellular milieu. The main secretion (Sec) pathway transports the majority of secretory proteins.

Its high capacity for protein export is heavily exploited in the enzyme-producing industry [3]. Importantly, the Sec pathway drives unfolded proteins across the plasma membrane. In contrast, the twin-arginine translocation (Tat) pathway has a relatively minor contribution in the overall secretion of proteins, but it has the fascinating ability to transport large, already folded and co-factor containing proteins across the *Bacillus* membrane.

Over the years, *B. subtilis* has been engineered to produce various proteins and compounds that are important industrially, pharmaceutically and/or in our daily life [1,11]. Initially, this involved mostly random mutagenesis and selection procedures, however, more recently, the rational modification of known regulatory mechanisms and pathways has gained momentum, thereby expanding the portfolio of proteins and metabolites that can be produced by the *B. subtilis* cell factory. In this review, we describe examples of *B. subtilis* cell engineering for the production of a range of high-value compounds, with particular focus on secreted proteins and inositol stereoisomers.

2. Protein Secretion

2.1. Two secretion pathways in *B. subtilis*

2.1.1. Sec pathway

In industrially optimized fermentations, *B. subtilis* production strains can secrete proteins to levels of more than 25 g/L of culture [3]. Such fermentations are commercially viable due to the possibility of bulk production on cheap media and downstream processing of secreted proteins at relatively low costs. Intriguingly, the mass secretion of proteins is achieved by a single pathway, the Sec pathway, which not only secretes proteins of interest at high levels, but which also facilitates membrane protein biogenesis and the secretion of native proteins that are involved in processes ranging from nutrient acquisition, chemotaxis, and stress management to cell wall regeneration [12]. Most likely, the Sec pathway has already been tuned for enzyme secretion during the evolution of *B. subtilis*, which has taken place in the soil and plant rhizosphere [3]. In its natural habitat, *B. subtilis* feeds on organic matter derived from plants and animals. To make use of this decaying biomass, *B. subtilis* has to secrete enzymes that convert polysaccharides into smaller sugars, and proteins into peptides and amino acids, that can subsequently be taken up by the bacterium. Clearly, to thrive in this ecological niche, and to successfully compete with other organisms for the available nutrients, a high-capacity machinery for enzyme secretion is advantageous. This is leveraged by the *Bacillus* Sec pathway, which entails a highly efficient machinery to target newly synthesized proteins from the ribosome to the membrane, to push them through the membrane-embedded Sec channel, and to fold them on the *trans*-side of the membrane. Subsequently, the exported proteins can diffuse into and across the cell wall, which is composed of peptidoglycan and other polymers, *e.g.* (lipo-)teichoic acids. As a consequence of the porous structure of the cell wall and the absence of an outer membrane, proteins that have been translocated over the *Bacillus* cytoplasmic membrane will be secreted into the extracellular milieu, unless they are specifically retained in the membrane or cell wall by particular anchoring mechanisms [12].

Importantly, in absence of an outer membrane, *B. subtilis* and its products are naturally free of endotoxins. This makes them safe for human use, consumption and health-related purposes, a feature acknowledged by the United States Food and Drug Administration (FDA) through the granting of the Generally Recognized as Safe (GRAS) status to many products of *B. subtilis* [13-15]. For the same reason, the European Food Safety Authority (EFSA) has earmarked *B. subtilis* for the Qualified Presumption of Safety (QPS) status [16]. These favorable traits of *B. subtilis* are complemented by the fact that the secreted enzymes produced with this bacterium can be applied in environmentally acceptable and sustainable ('green') alternatives to hazardous and energy-consuming chemical processes, the production of biofuels, and in a plethora of other industrial processes.

A principal feature of the Sec pathway is that it converts metabolic energy in the form of ATP and the proton-motive force (pmf) into a mechanical force, which drives proteins through the membrane-embedded Sec channel. This channel, which is composed of the SecY, SecE, and SecG proteins, permits the membrane passage of proteins that differ widely in size and amino acid composition [12]. The ATPase SecA serves as a cytoplasmic force-generator that accepts and drives secretory precursor proteins through the Sec channel in cycles of ATP binding and hydrolysis, whereas the membrane-anchored SecDF protein uses the pmf to enhance the efficiency of protein translocation [17-19].

At the cytoplasmic end of the Sec channel, the signal recognition particle (SRP) is believed to facilitate primarily the translocation of membrane proteins [20-22], whereas SecA and chaperones like CsaA or DnaK appear to be more important for facilitating this process for secretory precursor proteins [23-25]. At the extracytoplasmic side of the membrane, the post-translocational folding of various proteins emerging from the Sec channel is catalyzed by the peptidyl-prolyl cis/trans isomerase and chaperone PrsA [26-29], or the thiol-disulphide oxidoreductases (TDORs) BdbB, BdbC, and BdbD [30-32].

Of note, the specific targeting of proteins to the Sec channel is facilitated by their N-terminal signal peptides, which serve important roles especially in the initiation of Sec-dependent protein translocation [33]. Once the translocation process of a protein has been initiated, the signal peptide serves no further purpose and is removed by five different signal peptidases (SipS-W) that, in fact, liberate the fully translocated protein from the membrane [34-36]. Once cleaved, the signal peptide is degraded by signal peptide peptidases. The main signal peptide peptidase of *B. subtilis* is probably the intra-membrane cleaving protease RasP [37,38]. Other proteases, known as SppA and TepA, have also been implicated in signal peptide degradation, but they seem to have only minor roles in this process, if any [39,40].

Three proteases, named HtrA, HtrB, and WprA, serve in the quality control of newly folded translocated proteins [41-44]. As part of the extracytoplasmic protein quality control machinery, the two-component regulatory system CssRS senses the presence of misfolded secretory proteins at the membrane-cell wall interface. In case the cell suffers from this so-called secretion stress, the CssRS system triggers expression of the *htrA* and *htrB* genes [41,45,46]. Subsequently, the enhanced production of HtrA and HtrB leads to removal of the CssRS-inducing cue. Further, the WprA protease is responsible for a significant part of the protein degradation that occurs at the membrane-cell wall interface, allowing protein quality control at the exit of the Sec channel [47-51]. Importantly, not only proteinaceous factors, like PrsA and the Bdb proteins, facilitate the folding of translocated proteins. Also, the negatively charged cell wall polymers, such as the afore-mentioned (lipo-)teichoic acids, contribute to protein folding, possibly by forming a reservoir of cations that are major facilitators of post-translocational secretory protein folding [52-54].

2.1.2. Twin-arginine translocation pathway (Tat pathway)

In contrast to the Sec pathway, the Tat pathway exports fully folded proteins that often contain cofactors [55-57]. This pathway derived its name from the fact that the signal peptides that target proteins to the membrane-embedded Tat translocase contain an N-terminal S/T-R-R-X-F-L-K consensus motif, which includes adjacent 'twin-arginine' residues [58,59]. Both the folded state of the cargo protein and the twin-arginine signal peptide are essential for export of proteins via Tat. The Tat pathway of *B. subtilis* includes two parallel-acting translocases, TatAyCy and TatAdCd, each of which consists of only two components generally known as TatA and TatC [60,61]. The TatAyCy core translocase of *B. subtilis* is constitutively expressed and it consists of the TatAy and TatCy proteins [62,63]. This translocase targets the Rieske iron-sulfur protein QcrA to the cytoplasmic membrane [64,65], the metallophosphoesterase YkuE to the cell wall [66], and the peroxidase EfeB to the membrane-cell wall interface and the extracellular milieu [62,67]. In addition, the TatAdCd translocase, which consists of the TatAd and TatCd proteins, is expressed exclusively upon phosphate starvation [68]. The only currently known substrate of TatAdCd is the phosphodiesterase PhoD [60,62]. *B. subtilis* also contains a third TatA protein, named TatAc, which was shown to enhance protein export by TatAyCy. However, TatAc is dispensable for protein export via the *Bacillus* Tat pathway [69]. Judged by the general conservation of the Tat pathways encountered in bacteria, archaea and plant chloroplasts, and the results of studies performed in these organisms and organelles, it is believed that in *Bacillus* species a membrane-embedded complex of TatA and TatC recruits pre-folded cargo proteins in the cytoplasm [57,70]. Upon recruitment of oligomeric TatA complexes, the bound cargo protein will be translocated across the membrane and its signal peptide will be cleaved by one of the five signal peptidases of *B. subtilis* [62,64,71]. The Tat pathway holds great promise for heterologous protein production as has been shown by studies in *Escherichia coli* [72,73], where, for example, antibody fragments and the human growth hormone could be secreted via Tat to high levels [74,75]. In principle, this should also be possible via the Tat pathway of *B. subtilis*, as is indicated by the rerouting of some normally Sec-dependently secreted proteins for secretion via Tat [76-78]. However, the yields were so far low and many attempts were unsuccessful [79]. Most likely this relates to very tight quality control of the folding state of proteins targeted to Tat and, possibly, competition from the highly effective Sec machinery, which accepts also heterologous proteins with twin-arginine signal peptides [77]. It is, therefore, still a great challenge to unlock the *Bacillus* Tat pathway for protein production.

2.2. Engineering the Sec secretion pathway

It is an ambitious enterprise to make the already very effective Sec secretion machinery even more productive by cell factory engineering. Nonetheless, over the years various examples have been documented in the literature, which are presented in the following paragraphs. The reason why cell factory engineering can facilitate enhanced protein secretion relates to the fact that, for different secretory proteins, different bottlenecks can be encountered on the path from the ribosome through the Sec channel and the cell wall, and into the growth medium [40,80].

2.2.1. Chaperones

Engineering the *B. subtilis* chaperone machinery in the cytoplasm has been tried extensively. Although this is a

logical approach, not many successful examples have been documented. An early example concerns the co-expression of the *E. coli* chaperone SecB in *B. subtilis*, as this chaperone is absent from *Bacillus* species [81]. In addition, the SecA protein of *B. subtilis* was provided with a C-terminal SecB-binding domain derived from *E. coli* SecA. Indeed, this resulted in improved secretion of two model proteins, namely the *E. coli* maltose-binding protein MalE11 and the alkaline phosphatase PhoA [82]. Alternatively, engineering of the C-terminal domain of SecA allowed over two-fold improved secretion of human interferon-α2b [83].

2.2.2. Sec channel components

Engineering of the Sec channel is also a seemingly logical choice to enhance the protein secretion capacity of *B. subtilis*. However, this turned out to be challenging. In a recent study, the contributions of non-essential Sec pathway components of *B. subtilis* to the secretion of three highly expressed industrial enzymes was evaluated, namely the α-amylases AmyE from *B. subtilis* and AmyL from *Bacillus licheniformis*, and the serine protease BPN' from *Bacillus amyloliquefaciens* N' [40]. This showed that SecDF and SecG could be good targets for Sec channel engineering. Indeed, it has been shown that overexpression of *secDF* can facilitate improved secretion of AmyL and AmyS from *Geobacillus stearothermophilus* [84]. On the other hand, there is no published evidence that an altered expression level of *secG* could improve protein secretion in *B. subtilis* [84,85].

2.2.3. Signal peptidases

Since signal peptidases facilitate the release of translocated proteins from the cytoplasmic membrane, it is conceivable that they may represent a secretion bottleneck. This idea was also supported by the finding that *B. subtilis* has five signal peptidase genes, suggesting that this bacterium could face a shortage of such enzymes under particular conditions. Indeed, overexpression of the signal peptidase SipS was shown to lead to enhanced precursor processing kinetics in *B. subtilis* [86,87]. Unexpectedly, improved processing rates of the α-amylase AmyQ were also observed upon deletion of the signal peptidases SipS or SipU [34,86,88-90]. This suggests that, in wild-type *B. subtilis*, the paralogous signal peptidases may compete for binding and cleavage of particular precursor proteins [34,89]. Thus, not only the overexpression of secretion machinery components can lead to the removal of secretion bottlenecks, but also the deletion of particular redundant components.

2.2.4. The intramembrane-cleaving protease RasP

One of the most drastic improvements in protein secretion was achieved through overexpression of the intramembrane protease RasP [38]. This led to significantly enhanced production of two difficult-to-produce enzymes, namely a serine protease from *Bacillus clausii* that was overproduced about 2.5-fold, and the α-amylase AmyAc from *Paenibacillus curdlanolyticus* that was overproduced up to 10-fold under conditions that mimic industrial fermentation [38]. These findings suggest that, especially in case of difficult-to-produce proteins, it is important to keep the membrane free from accumulating processed signal peptides and/or malfolded precursor proteins.

2.2.5. The extracytoplasmic folding catalyst PrsA

The lipoprotein PrsA has both peptidyl-prolyl cis/trans isomerase and chaperone activity. Its critical function in the post-translocational folding of various secreted proteins has made PrsA a focal point of many efforts to engineer *B. subtilis* for improved secretory protein production [28]. Thus, it was shown that overexpression of PrsA can be very beneficial not only for the production of enzymes from *Bacillus* species, such as amylases and proteases [28,43, 84,91], the esterase LipA [92], or γ-glutamyltranspeptidase [93], but also for a lipoxygenase from *Anabaena* [94]. Moreover, it was reported that enhanced expression of PrsA in *B. subtilis* can increase the production of antigens from pathogenic bacteria, like the pneumolysin from *Streptococcus pneumoniae* [43], and pharmaceutically relevant proteins, like single-chain antibodies [95-97], or human interferon-β [98]. A complementary strategy involved the heterologous co-expression of a protein of interest with the 'cognate' PrsA from the respective species. The latter approach was first explored for expression of the protective antigen of *Bacillus anthracis* in *B. subtilis*, which led to substantially enhanced production levels of this protein [99]. More recently, it was demonstrated that the secretory production of various heterologous α-amylases in *B. subtilis* was substantially increased by co-expression of the PrsA from the same species from which the respective α-amylase was derived [100]. In addition, this study demonstrated that it is possible to engineer the PrsA of *B. licheniformis* to achieve further improvements in the α-amylase yield. Thus, it seems that there is substantial benefit in identifying the optimal pairing of secretory proteins of interest with particular PrsA proteins, and in the fine-tuning of PrsA expression levels [100].

2.2.6. Thiol-disulphide oxidoreductases

Disulphide bond formation in bacteria, archaea and eukaryotes is catalyzed by thiol-disulphide oxidoreductases (TDORs). These enzymes employ a universally conserved mechanism for stabilizing extracytoplasmic proteins that is

based on the oxidation of two cysteine thiol groups. Although this process can occur spontaneously, efficient disulphide bonding *in vivo* is catalyzed by TDORs [101]. On the extracytoplasmic side of the membrane, which is an oxidizing environment, TDORS usually act as thiol oxidases to form disulphide bonds in proteins that are exported from the cytoplasm. The best-characterized thiol oxidases of *B. subtilis* are BdbB, BdbC, and BdbD, which have complementary modular functions in the folding of a few native extracytoplasmic proteins of *B. subtilis* [102-104]. BdbC and BdbD were shown to be required for efficient heterologous secretion of the *E. coli* alkaline phosphatase PhoA [30,105]. However, it turned out to be difficult to engineer these Bdb proteins for optimized heterologous protein production, which may relate to the fact that they can cause oxidative misfolding of apocytochrome c thereby precluding proper haem insertion [106,107]. Interestingly, this hurdle can be overcome by heterologous expression of the DsbA lipoproteins of *Staphylococcus aureus* [30] or *Staphylococcus carnosus* [31]. This may relate to the fact that the activities of these DsbA proteins depend on redox-active medium components, rather than a quinol oxido-reductase in the membrane as is the case for BdbD. An additional increase in the production of PhoA could be achieved through depletion of the major cytoplasmic thiol reductase TrxA, which diminishes the overall protein-reductive power of *B. subtilis* [31]. Together, these findings imply that the engineering of *Bacillus* strains with enhanced properties for the secretion of disulphide-bonded proteins is feasible [30-32].

2.2.7. Extracellular proteases

Once a protein has been secreted effectively, it can still be degraded by extracellular proteases. This appears to be one of the most serious bottlenecks in protein production in *B. subtilis* [10]. In particular, the successive deletion of protease genes from *B. subtilis* strains resulted in substantial improvements in productivity of enzymes and pharmaceutical proteins [97,108-111]. To date, strains lacking up to 10 genes for extracytoplasmic or secreted proteases have been reported [112]. Overall, the deletion of protease genes enhances the secretion of intrinsically protease-sensitive or slowly folding proteins, on the one hand by reduced degradation, but perhaps also because the protease mutant strains accumulate the folding catalyst PrsA to higher levels [113]. Yet, the gain in productivity comes at the expense that the growth medium contains many cytoplasmic and membrane proteins, and that more extensive product purification will be necessary [50]. Most likely, this relates to the fact that the secreted proteases degrade cellular proteins from dead cells and, also, that the secreted proteases normally control the level of autolysin activity [50,114]. Thus, deletion of protease genes may lead to enhanced autolysis.

2.2.8. Genome reduction

In line with the deletion of particular gene functions, like protease genes, the deletion of redundant genome sequences was shown to improve the production of some proteins [115-119]. Possibly, the deletion of up to 36% of the *B. subtilis* genome in the PG10 strain makes translation of difficult-to-produce target proteins more efficient, whereas there may be less competition for Sec translocons between proteins of interest and the native secretory proteins. Moreover, proteolysis is severely reduced as exemplified for certain staphylococcal antigens [51,119]. A downside of the current genome-minimized strains is that their growth is poor compared with the parental strains [51,120,121].

3. Metabolic Engineering of *B. subtilis*

B. subtilis has served as a successful platform to produce various metabolites with commercial, pharmaceutical, and industrial values. Some selected examples are presented in the following paragraphs.

3.1. para-Aminobenzoic acid

para-Aminobenzoic acid, a precursor to produce aromatic polymers, was produced from the shikimate pathway through metabolic engineering of *B. subtilis*. A strategy was reported, including repair of the defective indole-3-glycerol phosphate synthase gene (*trpC2*) in the parental strain 168, knockout of the gene for one of the chorismate mutase isozyme (*aroH*), and overexpression of genes for the aminodeoxychorismate synthase (*pabAB*) from *Corynebacterium callunae* and aminodeoxychorismate lyase (*pabC*) from *Xenorhabdus bovienii* [122]. Furthermore, an artificial fusion-enzyme (PabABC) was created for channeling the carbon flux. It was found that product toxicity was the overall limiting factor, and surprisingly the performance in the designed biochemical pathway was less important. The highest titer of para-aminobenzoic acid that was achieved was 3.22 g/L [122].

3.2. 2,3-Butanediol

2,3-Butanediol is a promising alternative for biofuel production. However, since most of the 2,3-butanediol producers are opportunistic pathogens, they are not suitable for industrial scale fermentation. As indicated above, *B. subtilis* generates d-(−)-2,3-butanediol (purity > 99%) only under conditions low oxygenation. *B. subtilis* was therefore engineered to produce chirally pure meso-2,3-butanediol as follows [123]. The *bdhA* gene encoding d-

(−)-2,3-butanediol dehydrogenase was deleted, and *acoA* was knocked out to avoid the degradation of acetoin, which is the precursor of 2,3-butanediol. In addition, both the *pta* and *ldh* genes were deleted to decrease the formation of acetate and L-lactate byproducts. Furthermore, the *budC* gene from *Klebsiella pneumoniae*, encoding the meso-2,3-butanediol dehydrogenase, was introduced and the native *alsSD* gene was overexpressed to enable the efficient production of chiral meso-2,3-butanediol. Finally, the *udhA* gene, encoding a soluble transhydrogenase, was overexpressed to increase the pool of NADH, which is needed to facilitate the conversion of meso-2,3-butanediol from acetoin. Cultivating the engineered *B. subtilis* cells under limited oxygen conditions, 103.7 g/L of meso-2,3-butanediol was produced, and the titer of the main byproduct, acetoin, was as low as 1.1 g/L.

3.3. Ethanol

B. subtilis is a facultative anaerobe, although it was generally considered as an obligate aerobe until 1998. It is thus able to ferment glucose to produce lactate and butanediol (see above), but not to produce ethanol or acetate. In order to construct an artificial ethanologenic *B. subtilis* strain, the *ldh* gene encoding the native lactate dehydrogenase was replaced with an artificial operon made of the *pdc* and *adhB* genes of *Zymomonas mobilis*, encoding pyruvate decarboxylase and alcohol dehydrogenase II, respectively [124]. The engineered *B. subtilis* strain was found to produce not only ethanol, but also butanediol. To shut off the butanediol production, the *alsS* gene for acetolactate synthase was inactivated. In such a way, ethanol production was enhanced up to 89% of the theoretical yield. The *udhA* gene from *E. coli* encoding transhydrogenase was expressed to improve the cell growth rate and to ensure early onset of ethanol production. Long-term cultivation of thus engineered *B. subtilis* achieved 8.9 g/L of ethanol production [124].

3.4. D-Lactic acid

Poly lactic acid is a renewable and biodegradable plastic, and currently most of the poly lactic acid produced is composed of L-lactic acid. The copolymer of the L- and D-stereoisomers of lactic acid was shown to have improved physical properties and, accordingly, it is expected that its use will be expanded [125]. Therefore, an efficient procedure to obtain D-lactic acid was considered desirable. To achieve this objective, a *B. subtilis* strain was metabolically engineered to produce D-lactic acid. Since *B. subtilis* does not possess a *ldhA* gene for D-lactate dehydrogenase, each one of nine heterologous *ldhA* genes from *Bacillus coagulans* and *Lactobacillus delbrueckii* was introduced to evaluate the D-lactic acid productivity [126]. Among these, a strain expressing the *ldhA* gene from *L. delbrueckii* subspecies *bulgaricus* exhibited the highest D-lactic acid titer (about 1 M). These results showcase the potential of *B. subtilis* as a platform organism for synthesis of various compounds at minimal process costs.

3.5. Menaquinone-7

Menaquinone-7, a vitamin K-related compound, holds promise for the prevention of osteoporosis and cardiovascular calcification. Therefore, a strain of *B. subtilis* was engineered for high-titer production of menaquinone-7 [127]. The menaquinone-7 biosynthesis in *B. subtilis* involved five modules, namely glycerol dissociation, and the shikimate, pyrimidine metabolic, methylerythritol phosphate, and menaquinone-7 pathways. Production of the GlpK and GlpD proteins in the glycerol dissociation pathway were enhanced to give about 10% increase in the menaquinone-7 titer. On the other hand, the *mgsA* and *araM* genes were deleted to increase menaquinone-7 production by 15%. In addition, *aroGD146N* in the shikimate pathway, *pyrGE156K* in the pyrimidine metabolic pathway, *hepS* in the methylerythritol phosphate pathway, and *vgb* were simultaneously overexpressed to increase the menaquinone-7 titer further. Finally, a recombinant strain was constructed that overexpresses all the genes including *glpK*, *glpD*, *aroGfbr*, *pyrGfbr*, *hepS*, and *vgb*, whereas *mgsA* and *araM* were inactivated. The resulting menaquinone-7 titer reached up to 300 mg/L.

3.6. Riboflavin

Riboflavin, also known as vitamin B2, is an essential nutrient for humans and animals that must be obtained from the diet. Therefore, it is often added in food and feed as a dietary supplement. To support its mass consumption, riboflavin has been produced industrially and its production already has a decades-long history. It was once produced by an organic chemical synthesis process. However, nowadays, the fermentation method, which is superior in terms of economy and environmental sustainability, has become the mainstream production method involving engineered strains of *B. subtilis* and *Ashbya gossypii*. The history of the development of strains for riboflavin overproduction and future prospects for further improvements are described in detail in a separate review [128].

3.7. Taxol

Taxol belongs to the terpenoids, which are well known for their medicinal and commercial applications. In order to engineer *B. subtilis* as a cell factory for the production of taxol, the plant-derived taxadiene synthase (TXS) enzyme was introduced [129]. TXS is a key enzyme for the conversion of the precursor geranylgeranyl pyrophosphate to taxa-

4,11-diene. The other genes encoding eight enzymes in the biosynthesis pathway for taxol were also overexpressed to increase the flux of the precursor, by introducing a synthetic operon containing the genes encoding the 2-*C*-methyl-D-erythritol-4-phosphate pathway (*dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, and *ispG*) together with *ispA* encoding geranyl and farnesyl pyrophosphate synthases to provide farnesyl pyrophosphate. Moreover, the *crtE* gene of *Pantoea ananatis*, which encodes geranylgeranyl pyrophosphate synthase was additionally introduced to increase the supply of the precursor. The overexpression and introduction of these genes led to a significant increase in production of taxadiene compared to the previous strain only expressing TXS [129].

3.8. Engineering inositol metabolism in *B. subtilis*

Inositol, cyclohexane-1,2,3,4,5,6-hexol, stands for a group of sugar alcohols with half the sweetness of sucrose. Epimerizing the six hydroxyl groups, there are nine stereoisomers of inositol, and *myo*-inositol is the most abundant in nature, often found in the brain and other mammalian tissues. *myo*-inositol is known to mediate various cell signal transduction pathways, playing an important role as the structural basis for various inositol phosphates that serve as secondary messengers in eukaryotic systems [130]. In addition, *myo*-inositol is a component of phosphatidylinositol phosphate lipids contained in cell membranes. Consequently, inositol associated with lipids is found commonly in many foods [131]. In plants, the hexaphosphate of *myo*-inositol, phytic acid, serves as a phosphate store that accumulates in nuts, beans, and cereals with high bran content. *myo*-inositol was previously considered to belong to the vitamin B complex and also referred to as vitamin B8. However, it was subsequently shown that *myo*-inositol is not an essential nutrient, because it is produced in the human body from glucose [132].

Besides *myo*-inositol, the other stereoisomers naturally occurring are *scyllo*-, *muco*-, D-*chiro*-, and *neo*-inositol. The occurrence of these compounds is rare in nature. However, some of them have specific and beneficial biological activities. *scyllo*-inositol was found to prevent the development of amyloid-beta plaques in the brains of transgenic mice, and it was shown to reverse memory deficits, and also to effectively reduce the other associated symptoms. Therefore, *scyllo*-inositol has been regarded as a promising medicine for the treatment of Alzheimer's disease [133]. On the other hand, D-*chiro*-inositol and its 3-*O*-methyl derivative, D-pinitol, have an insulin-mimetic activity in lowering the blood glucose levels [134]. Therefore, the latter compounds have been studied for their possible application in the treatment of diabetes mellitus and the polycystic ovary syndrome [135]. To develop cell factories that can produce the rare inositol stereoisomers, the inositol metabolism in *B. subtilis* has been engineered as described below.

3.8.1. Inositol metabolism in B. subtilis and conversion of inositol stereoisomers

B. subtilis utilizes inositol stereoisomers, including *myo*-inositol, D-*chiro*-inositol, and *scyllo*-inositol as carbon sources [136]. The *iolABCDEFGHIJ* operon is responsible for a complete set of enzymes to catabolize *myo*- and D-*chiro*-inositol [137]. The *iolF* and *iolT* genes encode two inositol transporters to take up *myo*- and D-*chiro*-inositol, respectively [136,138]. The *iolG* gene encodes *myo*-inositol dehydrogenase, which converts *myo*-inositol to *scyllo*-inosose with reduction of NAD^+ as the first reaction in the catabolism [139]. IolG can also react with D-*chiro*-inositol, but not at all with *scyllo*-inositol, because of its strict preference of the axial hydroxyl group as a target [140]. Both the promoters of the *iol* operon and *iolT* are under regulatory control of the IolR transcriptional repressor, whose DNA binding is antagonized by 2-deoxy-5-keto-gluconic acid-6-phosphate, the product of the IolC kinase [141-143]. There are two other inositol dehydrogenases, IolX and IolW, which are specific for *scyllo*-inositol and require NAD^+ and $NADP^+$, respectively [143]. Each of them converts *scyllo*-inositol to *scyllo*-inosose, which is the same product that is also produced by IolG. IolU was identified as the third *scyllo*-inositol dehydrogenase, but the physiological significance of this enzyme remains unclear, since it only reduces *scyllo*-inosose into *scyllo*-inositol in an NADPH-dependent manner [144]. The *iolX* gene is induced only when *scyllo*-inositol is present as the sole carbon source [136]. In contrast, *iolW* is almost constitutively expressed, but it does not help growth on *scyllo*-inositol [136]. Therefore, it can be concluded that IolX is responsible for *scyllo*-inositol catabolism, and IolW and IolU may function for another purpose, such as the generation of *scyllo*-inositol.

Notably, *iolI* was found to encode an inosose isomerase, which converts *scyllo*-inosose to 1-keto-D-*chiro*-inositol that can serve as an additional substrate of IolG to be converted to D-*chiro*-inositol [141]. Actually, the combination of IolG and IolI permits the conversion of *myo*-inositol into D-*chiro*-inositol as described in the next section.

3.8.2. D-chiro-Inositol

Although it has not been fully elucidated how it works, D-*chiro*-inositol has a pharmaceutical value for the treatment of diabetes mellitus and the polycystic ovary syndrome. It was shown to improve the efficiency of insulin and also promotes ovulation. *B. subtilis* was engineered to produce D-*chiro*-inositol from *myo*-inositol [140]. In the first and second steps of the *myo*-inositol catabolic pathway, *myo*-inositol is converted to *scyllo*-inosose by IolG and then to 3d-(3,5/4)-trihydroxycyclohexane-1,2-dione by IolE. As

described above, *iolI* encodes an inosose isomerase, which converts *scyllo*-inosose into 1-keto-D-*chiro*-inositol, and it was found that IolG reduces 1-keto-D-*chiro*-inositol into D-*chiro*-inositol. The *iolE* gene was inactivated to block the *myo*-inositol catabolic pathway to accumulate *scyllo*-inosose, which was further converted into D-*chiro*-inositol by IolI and IolG to enable conversion of at least 6% of the input *myo*-inositol into D-*chiro*-inositol.

3.8.3. scyllo-Inositol

As described above, *scyllo*-inositol has been regarded as a promising therapeutic agent for Alzheimer's disease. *B. subtilis* cell factories have been developed with modified inositol metabolism that converts *myo*-inositol into *scyllo*-inositol in the culture medium. The first-generation cell factory for this process was constructed by deleting the three genes *iolR*, *iolX*, and *iolI* and by introducing the missense mutation *iolE41*. The aim of these mutations was to make the expression of the *iolABCDEFGHIJ* operon including *iolG* constitutive, and to abolish dehydrogenation of *scyllo*-inositol as well as isomerization and dehydration of *scyllo*-inosose. In this cell factory, *scyllo*-inositol production was achieved by conversion of nearly half of the initial *myo*-inositol amount (10 g/L) after 72 h cultivation, but the other *myo*-inositol was consumed [136]. The second-generation cell factory was constructed by combining deletions of *iolR*, *iolX*, and *iolABCDEFHIJ* and simultaneous overexpression of *iolG* and *iolW* [145]. In this case, the initial 10 g/L of *myo*-inositol was completely converted to *scyllo*-inositol within 48 h. However, when the initial concentration of *myo*-inositol was increased to 50 g/L, the conversion was yet limited to produce 15.1 g/L of *scyllo*-inositol. Therefore, a third-generation cell factory was constructed by additional overexpression of *iolT* and *pntAB*, the major transporter of *myo*-inositol and the membrane-integral nicotinamide nucleotide transhydrogenase of *E. coli*, respectively. However, the conversion efficiency was not improved dramatically. Nonetheless, it was found that an increased concentration of Bacto soytone in the culture medium up to 4% enhanced the conversion, and as a result, the cell factory was improved to yield a *scyllo*-inositol production rate of 27.6 g/L in 48 h [146].

3.8.4. Production of scyllo-inositol from glucose

In natural biological systems, *myo*-inositol biosynthesis is conserved, including the phosphorylation of glucose into glucose-6-phosphate, conversion of glucose-6-phosphate into *myo*-inositol 1-phosphate by the *ino1*-encoded *myo*-inositol-1-phosphate synthase, and by removal of a phosphate from *myo*-inositol-1-phosphate to form *myo*-inositol by the inositol monophosphatase. The key enzyme *myo*-inositol-1-phosphate synthase (MI1PS) is found in some archaea and bacteria, including *Mycobacterium tuberculosis*, which are capable of *myo*-inositol biosynthesis [147]. However, *B. subtilis* has no gene likely to encode this MI1PS, but it has the *yktC* gene encoding a functional inositol monophosphatase [148]. Therefore, *myo*-inositol biosynthesis in *B. subtilis* was expected to occur upon introduction of the *M. tuberculosis ino1* gene, and that *myo*-inositol would then be converted into *scyllo*-inositol as described above [136,145]. Thus, *M. tuberculosis ino1* was introduced into *B. subtilis*. The introduced enzyme was produced normally, but it failed to show the required activity in *B. subtilis*. Importantly, MI1PS requires NAD^+·NADH as an essential cofactor. Although the underlying mechanism remains to be elucidated, it was found that inactivation of *pbuE*, encoding a purine base/nucleoside efflux pump, resulted in a significant elevation in the intracellular levels of NAD^+·NADH. As a result, the introduced mycobacterial enzyme became active only upon the inactivation of *pbuE*, which resulted in the production of *myo*-inositol-1-phosphate, and the successive dephosphorylation into *myo*-inositol was catalyzed by an intrinsic inositol monophosphatase, YktC. The *myo*-inositol was subsequently isomerized into *scyllo*-inositol via the previously established artificial pathway involving the two inositol dehydrogenases, IolG and IolW, as described above. In addition, *glcP* and *glcK*, encoding a glucose transporter and a glucose kinase, respectively, were simultaneously overexpressed to feed more glucose-6-phosphate and accelerate *scyllo*-inositol production. Finally, an effective *B. subtilis* cell factory was constructed, which produced 2 g/L *scyllo*-inositol from 20 g/L glucose. This cell factory will hopefully help us to challenge the growing problem of Alzheimer's disease, by providing an inexpensive way to produce *scyllo*-inositol [149].

4. Conclusion and Future Perspectives

In the above sections, we have highlighted various examples of successful engineering of pathways for protein secretion and the production of high-value metabolites using *B. subtilis* as a cell factory. All improvements that were achieved in recent years have leaned heavily on so-called omics studies, which allowed us to disentangle many complex regulatory and functional cellular networks [1,3,63,150]. Advances in the omics area have been particularly instrumental in the rational design of strategies for cell factory engineering. However, despite many successes, the approaches applied for cell factory engineering can still be very cumbersome as they frequently rely to large extents on trial-and-error. Thus, there are still serious limits to the further development and optimization of potentially important new-generation *B. subtilis* cell factories to produce many different proteins,

enzymes and metabolites. These limitations reside mostly in understanding the dynamic regulation of *B. subtilis* metabolism in response to the stresses imposed by our engineering approaches, and in the best-possible ways to re-route cellular resources towards the desired products, be it secreted proteins, enzymes or metabolites. The many 'unknowns' in metabolic regulation in response to cell engineering for enhanced protein or metabolite production are the prime factors that nowadays restrict further optimization of *B. subtilis* towards super-producing cell factories, where the cellular growth and product synthesis are optimally balanced. Here, we foresee that genome-scale cellular modelling approaches, like resource balance analysis (RBA), which is a computational method based on resource allocation that can perform accurate quantitative predictions of whole-cell states [151-153], will help us to understand which metabolic pathways need to be tweaked in order to maximize the productivity for high-value proteins and metabolites by the *B. subtilis* cell factory. Engineering approaches guided by RBA can also be combined with high-throughput omics and nanoliter reactor-based screening technologies to achieve further improvements [154]. As such, the modification and optimization of this bacterium's metabolic circuits holds great promise to deliver many of the compounds that are urgently needed for a green and sustainable society, as well as a healthy human population.

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