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Recent Advances in Recombinant Protein Production by *Bacillus subtilis*

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Bacillus subtilis, recombinant protein production, origin strain, protein translocation, protein folding, proteolytic degradation

Abstract

Bacillus subtilis has become a widely used microbial cell factory for the production of recombinant proteins, especially those associated with foods and food processing. Recent advances in genetic manipulation and proteomic analysis have been used to greatly improve protein production in *B. subtilis*. This review begins with a discussion of genome-editing technologies and application of the CRISPR–Cas9 system to *B. subtilis*. A summary of the characteristics of crucial legacy strains is followed by suggestions regarding the choice of origin strain for genetic manipulation. Finally, the review analyzes the genes and operons of *B. subtilis* that are important for the production of secretory proteins and provides suggestions and examples of how they can be altered to improve protein production. This review is intended to promote the engineering of this valuable microbial cell factory for better recombinant protein production.

INTRODUCTION

The Gram-positive bacterial species *Bacillus subtilis* has been widely used for the industrial production of proteins because it is nonpathogenic, has a well-known genetic background, is amenable to high-density fermentation, and has excellent protein secretory capability (Harwood & Cranenburgh 2008). In recent decades, many strategies have been used to improve protein-expression levels in *B. subtilis*. Because the microenvironment of the host strain is critical for protein synthesis, translocation, and folding, the origin of the host strain significantly affects its protein-expression capability. The strain widely used in industrial production and academic research, *B. subtilis* 168, was obtained from *B. subtilis* Marburg through X-ray-induced mutation (Burkholder & Giles 1947). Unlike *B. subtilis* Marburg, *B. subtilis* 168 is an auxotroph that requires tryptophan and is unable to efficiently use amino acids of the glutamate family. The widely used protease-deficient strains WB600, WB700, and WB800 were derived from *B. subtilis* 168 through knockout of the genes encoding extracellular proteases (Wu et al. 1991, 2002; Ye et al. 1999). Recent studies that have focused on the modification of undomesticated *B. subtilis* strains for industrial production have produced strains that show great potential for improved protein expression (Kabisch et al. 2013, Liu et al. 2018, K. Zhang et al. 2016).

Once a promising origin strain has been chosen, it must be modified to satisfy the requirements of modern recombinant protein production, which involves multiple components related to protein expression (**Figure 1**). *B. subtilis* expresses intracellular chaperones that mediate the folding of

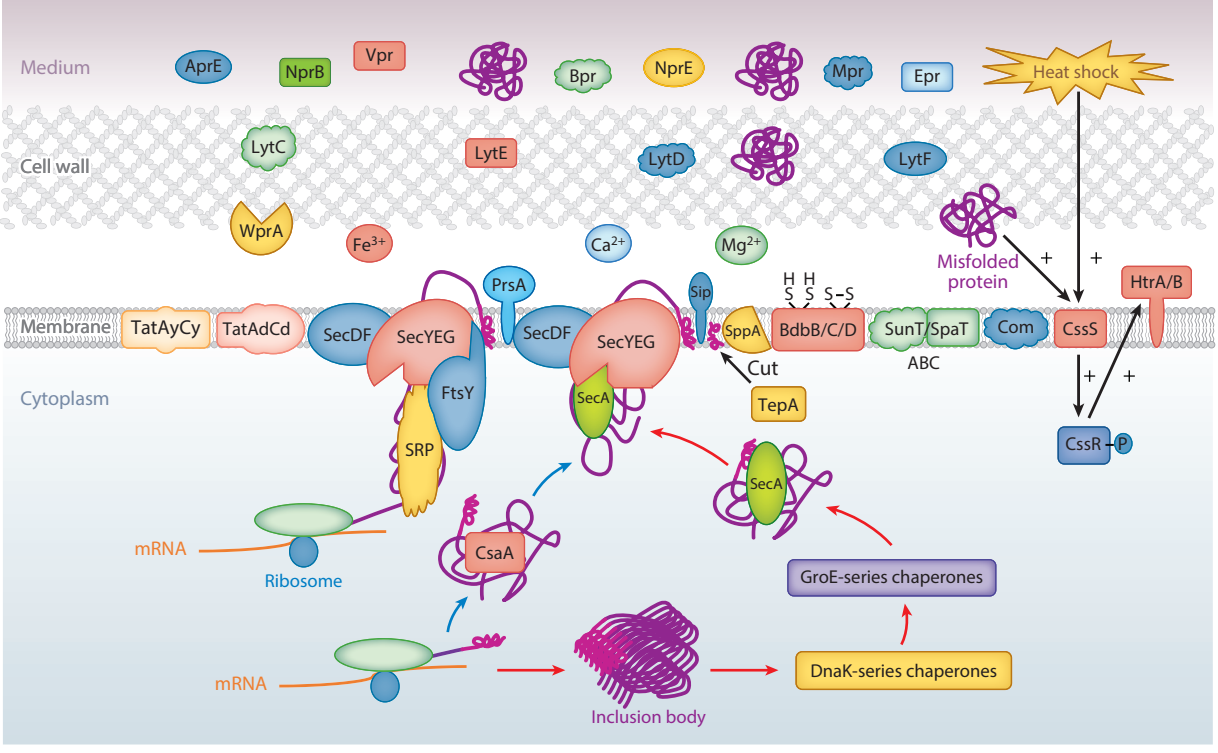

Figure 1

Schematic representation of the components involved in protein expression. These components include protein synthesis elements, chaperones, secretory translocases, and proteases. Abbreviation: SRP, signal recognition particle.

nascent polypeptides and intracellular protein oligomerization. They also maintain precursor proteins in a translocation-competent state and decrease precursor protein aggregation. Unlike the Gram-negative expression host *Escherichia coli*, *B. subtilis* lacks an outer membrane and associated periplasmic space, which gives it greater protein secretion capability. For efficient extracellular expression, efficient secretion must be coordinated with appropriate folding and degradation. The space between the cytoplasmic membrane and cell wall in *B. subtilis* is similar to the periplasmic space of Gram-negative bacteria. It is a crucial location where secretory proteins undergo folding to their mature form under strict quality control. The *B. subtilis* cytoplasmic membrane contains several types of proteins important for this process, including chaperone PrsA (Kontinen & Sarvas 1993); *Bacillus* disulfide bond (Bdb) formation proteins BdbB, BdbC, and BdbD; and quality-control proteases HtrA and HtrB (Bolhuis et al. 1999b). The cell wall also contains relevant proteins such as quality-control protease WprA and autolysins LytC, LytD, LytE, and LytF. Because the polymeric components of the *B. subtilis* cell wall are anionic, they have many bound cations (e.g., Ca^{2+}, Fe^{3+}, and Mg^{2+}) that are important for the folding and stability of secretory proteins (Chambert et al. 1990, Petitglatron et al. 1993). In addition to the critical components of its cytoplasmic membrane and cell wall, *B. subtilis* secretes seven extracellular proteases (NprE, AprE, NprB, Bpr, Mpr, Epr, and Vpr) that degrade extracellular proteins to different degrees, depending on their folding state and proteolytic sensitivity, for nutrient recycling. The levels/activities of all these critical components must be addressed to achieve optimal extracellular expression of appropriately folded, fully active recombinant proteins.

This review begins by introducing the recent advances in genome-editing technologies and discusses application of the CRISPR–Cas9 system to genome editing and transcriptional regulation. Developments in identifying the genomic heritage, provenance, and phenotype of several crucial legacy strains are summarized and followed by some suggestions about choosing an origin strain. Next, the factors involved in protein translocation and folding are introduced and strategies to modulate these factors for better protein expression are discussed. Finally, an analysis of proteolytic degradation of native and recombinant proteins is presented along with suggestions regarding suitable protease-deficient strains for different recombinant proteins and modification of the degree of cell lysis. The goal of this review of strategies to construct a better expression host is to help academic researchers and industrial producers achieve higher recombinant protein production. Greater production of various food-processing enzymes in *B. subtilis*, which have no food safety concerns, reduces the cost of corresponding food industries and promotes their development.

GENOME-EDITING TECHNOLOGIES

Traditional strategies for genetic manipulation in *B. subtilis* are based on homologous recombination. These genome-editing methods include the use of antibiotic-resistance markers, counterselectable markers, and the cre/*loxP* system (Yan et al. 2008). Recently, the CRISPR–Cas9 system, which is derived from the bacterial adaptive immune system, has been adapted for the genetic manipulation of many prokaryotic and eukaryotic organisms. The CRISPR–Cas9 system is highly efficient and its use saves time, and the modified organism has no marker. Three different CRISPR–Cas9 systems have been used in *B. subtilis*: a single-plasmid system, a double-plasmid system, and a chromosomal system (Altenbuchner 2016, So et al. 2017, Westbrook et al. 2016). With the assistance of a single-guide RNA (sgRNA), the Cas9 protein can introduce a double-strand break (DSB) at a target site within the *B. subtilis* genome. A homologous repair template then directs homologous recombination, introducing a gene disruption, deletion, or insertion. In addition to the often-used *Streptococcus pyogenes* Cas9 (SpCas9), which recognizes the protospacer adjacent motif

(PAM) sequence NGG, in which N is any nucleotide, there are other potential CRISPR proteins with specific characteristics. The Cpf1 protein from *Francisella novicida* can introduce staggered DSBs instead of blunt-ended DSBs and can mediate efficient genome editing in *Corynebacterium glutamicum* (Jiang et al. 2017). A variant of the Cas9 from *F. novicida* recognizes a PAM sequence of CG or TG, which increases the choices of editing target (Hirano et al. 2016). Furthermore, through phage-assisted continuous evolution, an SpCas9 variant that recognizes the PAM sequences NG, GAT, and GAA and shows greater DNA specificity was obtained (Hu et al. 2018).

The Cas9 protein contains two catalytic residues, Asp 10 and His 840, required for nuclease activity. Mutation of either Asp 10 or His 840 to Ala yields the Cas9n protein, which can only cause a single break at the genome target, leading to little or no toxicity to the host bacterium compared with wild-type Cas9 (Ran et al. 2013). Mutation of both residues to Ala yields the dCas9 protein, which shows no nuclease activity, although it can still bind to the target site with the help of the sgRNA. Binding of the dCas9 protein at a specific site causes a DNA bubble that can interfere, through steric hindrance, with transcription by RNA polymerase (Peters et al. 2016). The dCas9 protein has been used to identify the targets of uncharacterized antibiotics and the essential gene network associated with cell growth. Furthermore, fusing RNA polymerase subunits α or ω to the dCas9 protein yields dCas9-α/ω, which shows an ability to repress or activate gene expression that depends on the distance from the target site to the transcription start site (Lu et al. 2019). Simultaneous repression or activation of the expression of multiple genes can be achieved by designing multilocus targeted sgRNA. Using dCas9-ω to simultaneously improve the expression of chaperone PrsA and repress the expression of proteases Bpr and Vpr improved the production of *Bacillus licheniformis* α-amylase (AmyL) by 250%. Combining the ability of dCas9-α/ω to activate or repress transcription with high-throughput screening allows the identification of crucial factors that limit recombinant protein expression (**Figure 2**). Once identified, the highly efficient gene insertion mediated by the CRISPR–Cas9 system can promote the directed evolution of these crucial factors. This is done by inserting variants obtained from completely random or semirational mutagenesis and using high-throughput screening techniques to identify the desired mutant strains.

IDENTIFICATION OF LEGACY STRAINS AND THE CONSTRUCTION OF ENGINEERED HOST STRAINS

The Genomic Heritage, Provenance, and Phenotype of Legacy Strains

The bacterial preservation centers around the world harbor numerous legacy *B. subtilis* strains, many of which were originally constructed from *B. subtilis* Marburg through transformation, transduction, domestication, or mutagenesis and exhibit genetic heterogeneity among their genomes (Zeigler et al. 2008). The genomic heritage and provenance of many legacy strains were never recorded or have been lost (Wahome & Setlow 2008). Legacy strains ATCC 6051 and NCIB 3610, the wildest Marburg isolates ever reported, were deposited in the American Type Culture Collection and the National Collection of Industrial Bacteria, respectively (Conn 1930). Meanwhile, there are five auxotrophic strains derived from *B. subtilis* Marburg by X-ray or UV mutagenesis, including *B. subtilis* 23, which requires threonine; *B. subtilis* 122, which requires nicotinic acid; and *B. subtilis* strains 160, 166, and 168, which require tryptophan (Burkholder & Giles 1947). Among them, 168 has been widely used in academic research and industrial production. A genome resequencing experiment revealed that 22 single-nucleotide polymorphisms exist between NCIB 3610 and *B. subtilis* 168, demonstrating the high level of identity they share (Zeigler et al. 2008).

The genetic heterogeneity among legacy strains is related to subtle differences in physiology and metabolism. Compared with the undomesticated strain ATCC 6051, *B. subtilis* 168 cannot

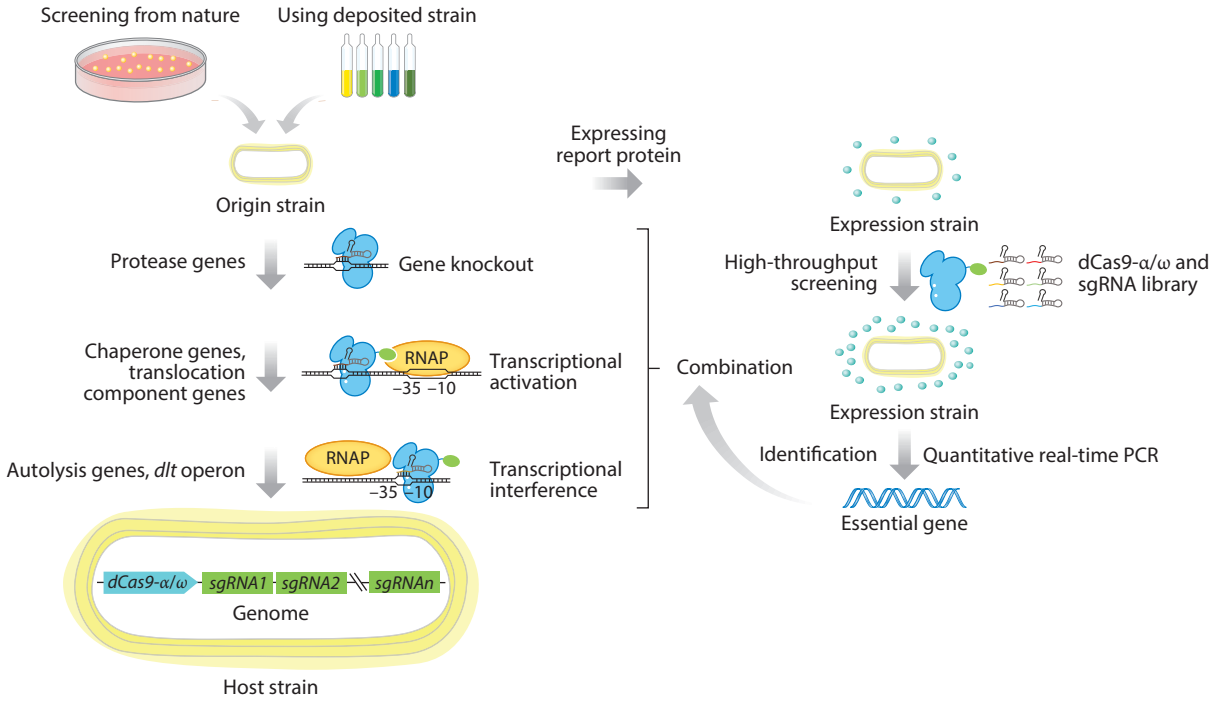

Figure 2

Construction of expression host strain through the CRISPR–Cas9 system in various combined strategies. Using the ability of dCas9-α/ω in repression and activation of gene expression, these strategies identify essential genes and regulate expression of multiple genes to increase protein expression capability of the host strain. Abbreviations: PCR, polymerase chain reaction; RNAP, RNA polymerase; sgRNA, single-guide RNA.

form swarm colonies because of the inactivation of *sfp* and *swrA*, a change that yields nonmotile multicellular chains during exponential growth (Chen et al. 2009). Similarly, the *gudB* gene of *B. subtilis* 168 encodes an intrinsically inactive glutamate dehydrogenase that contains a three-amino-acid (VKA) insertion (Belitsky & Sonenshein 1998). For that reason, *B. subtilis* 168 cannot efficiently utilize glutamate family amino acids (arginine, ornithine, and glutamine). In contrast, ATCC 6051 can efficiently utilize these amino acids and grow well in complex media containing components like soy peptone because its genome encodes an active glutamate dehydrogenase (Kabisch et al. 2013).

The transformation efficiencies of undomesticated strains, which are determined by their genotypes, are extremely low and that helps to stabilize their genomes. For example, the *comP* gene (part of the pathway that confers competence) of *B. subtilis* ATCC 6051 contains a frameshift mutation that results in early termination. The truncated sensor kinase ComP is missing its kinase domain and cannot activate the regulatory element ComA by changing its phosphorylation state (Kabisch et al. 2013). ComA is a transcriptional activator of competence determinants, and its inactivation decreases expression of competence determinants to a low level, yielding low transformation efficiency. Similarly, *B. subtilis* NCIB 3610 harbors an endogenous plasmid (pBS32) that encodes ComI, which inhibits competence through punctate colocalization with ComGA (Konkol et al. 2013). Mutation experiments have demonstrated that glutamine 12 of ComI is required for its interaction with ComGA, which causes ComGA mislocalization and leads to low transformation efficiency.

Converting an Undomesticated Strain into an Expression Host

Although the protease-deficient strains WB600, WB700, and WB800, derived from *B. subtilis* 168, have been widely used for industrial protein production, *B. subtilis* 168 is an auxotroph and cannot efficiently utilize glutamate family amino acids. Several undomesticated strains show growth properties more favorable than those of *B. subtilis* 168 as well as superior protein productivity. This suggests that undomesticated strains have great potential as either alternative industrial host strains themselves or the parents of alternative industrial host strains. *B. subtilis* ATCC 6051 has been modified through knockout of *lytC* and *spoIIGA* to construct an expression host strain that shows reduced autolysis and cannot form spores (Kabisch et al. 2013). Similarly, the disruption (using the CRISPR–Cas9 system) of *srfC*, *spoIIAC*, *nprE*, *aprE*, and *amyE* in the undomesticated strain *B. subtilis* ATCC 6051a created a superior expression host strain that forms much less foam, exhibits lower extracellular protease and α-amylase activities, and shows resistance to spore formation during the fermentation period (K. Zhang et al. 2016).

With the development of genome sequencing and metabolic analysis, differences in bacterial phenotype caused by genetic heterogeneity have been carefully investigated. Although the physiology and metabolic properties of bacterial strains can be largely optimized through genome editing, choosing the right origin strain is crucial for the construction of a strain suitable for industrial protein production. The right origin strain likely has favorable growth properties and protein-expression capability as well as fewer mutations in its protein-encoding genes, which can sometimes cause auxotrophy or subtle problematic phenotypes during protein production. The origin strain can be obtained by screening environmental bacteria for strains with superior growth and expression characteristics, or by using a strain deposited in an available collection that has a lineage similar to that of *B. subtilis* Marburg (**Figure 2**).

PROTEIN TRANSLOCATION AND FOLDING

Efficient protein secretion and folding are crucial during the production of recombinant proteins in *B. subtilis*. These processes are assisted by components of the translocation system as well as by both intracellular and extracytoplasmic chaperones (Harwood & Cranenburgh 2008). The level of heterologous protein production in *B. subtilis* is usually lower than that of homologous protein production (Li et al. 2004, Pohl & Harwood 2010). This might occur because the secretory and folding environments of the *B. subtilis* strains used for heterologous protein production are different than those of the parent strain, resulting in some bottlenecks. These secretion and folding bottlenecks may also exist during the production of some homologous proteins for the limitation of some crucial components of the protein-expression machinery. Therefore, the components of the secretion and folding machinery have been systematically studied to remove these bottlenecks.

Secretory Pathway and Signal Peptides

B. subtilis exports approximately 300 native proteins. These proteins perform physiological functions that include nutrient absorption, communication between cells, and detoxification for environmental competition (Tjalsma et al. 2004). *B. subtilis* contains at least four pathways for protein export through the cytoplasmic membrane, including the general secretion (Sec) pathway, the twin-arginine translocation (Tat) pathway, the pseudopilin export (Com) pathway, and the ATP-binding cassette (ABC) pathway (**Figure 1**) (Sarvas et al. 2004). The majority of secreted proteins transit the cytoplasmic membrane using the Sec pathway. During this process, the secretory proteins are loosely folded or unfolded while inside the cell and quickly fold into the correct conformation after translocation to avoid proteolytic degradation. In contrast, proteins that are

translocated through the Tat pathway are fully folded before translocation (Berks et al. 2003). Proteins translocated through either the Sec or Tat pathway are generally released into the extracellular medium, although some proteins that contain transmembrane segments or are lipoproteins can be exported and retained at the extracytoplasmic membrane surface. Proteins translocated through the Com pathway are usually required for the development of natural competence and retained at the cytoplasmic membrane, whereas a few specific proteins translocate through the ABC pathway (Tjalsma et al. 2004).

Most secretory proteins contain signal peptides, which can be divided into five groups (Sec, Tat, lipoprotein, Com, or ABC signal peptides) depending on their structure and export pathway. The first four types of signal peptide generally consist of three distinct domains (N, H, and C), whereas the ABC signal peptide does not contain domain H. The N domain, which is usually positively charged, contains at least one lysine or arginine residue that can interact with the translocation machinery and the negatively charged anionic polymer components of the membrane. The hydrophobic H domain contains a glycine or proline residue in the middle of a helical segment, creating a hairpin-like structure. The C domain contains a signal peptidase cleaving site for removing a signal peptide.

Sec or Tat signal peptides are commonly used for recombinant protein production in *B. subtilis*. Each secretory protein has a different optimal signal peptide. Numerous studies have been performed to identify the key features of these optimal signal peptides and dissect the relationships between these signal peptides and their corresponding secretory proteins. No notable characteristic has been found in these optimal signal peptides, and no clear correlations have been found between levels of secretory protein production and the length, N-terminal charge, hydrophobicity level, or discrimination score (calculated using the prediction tool SignalP) of these signal peptides (Brockmeier et al. 2006). However, studies of the expression of *Bacillus pumilus* BYG xylanase and *Fusarium solani pisi* cutinase in *B. subtilis* have suggested that the helical propensity of the signal peptide may play an important role in the secretion of proteins that contain less helical content (W. Zhang et al. 2016). Meanwhile, the levels of secretion obtained using different signal peptides showed a highly consistent trend when expression was mediated by promoters P_{glvm} and P_{43}.

Signal peptides from species closely related to *B. subtilis*, such as *B. licheniformis* and *Bacillus amyloliquefaciens*, can be used for protein secretion in *B. subtilis* (Degering et al. 2010, Rey et al. 2004). Because rational methods cannot be used to predict the optimal signal peptide for a secretory protein, efficient methods using high-throughput screening of signal peptide libraries have been developed. For example, high-throughput screening of a signal peptide library containing 173 *B. subtilis* signal peptides and 220 *B. licheniformis* signal peptides yielded a signal peptide that increased the production of *B. amyloliquefaciens* subtilisin BPN′ approximately sevenfold compared with that obtained using the wild-type signal peptide (Degering et al. 2010). In two separate studies, screening 173 *B. subtilis* signal peptides yielded constructs that increased the extracellular expression level of *Bacillus stearothermophilus* α-amylase (AmyS) by approximately 70% and 250%, respectively (Fu et al. 2018, Yao et al. 2019). After identifying a good signal peptide, secretory production can be further improved through saturation mutagenesis or randomized mutagenesis of the selected signal peptide (Caspers et al. 2010). For example, saturation mutagenesis of positions 2–7 in the N domain of the *B. subtilis* α-amylase (AmyE) signal peptide followed by high-throughput screening for variants with greatly improved cutinase activity identified four mutant strains that increased the activity by 200–300%. Interestingly, one of the variants showing increased activity (200%) exhibited lower secretion efficiency than the control strain, whereas another variant showing drastically decreased activity exhibited greater secretion efficiency than the control strain. It was speculated that excessive secretion can overload extracellular folding factors

like chaperone PrsA, which yields a high level of misfolded protein. The accumulation of misfolded protein upregulates expression of quality-control proteases HtrA and HtrB and results in increased protein degradation, which lowers the cutinase expression level. Meanwhile, the signal peptide was speculated to be involved in protein synthesis through its influence on mRNA stability or secondary structure. Overall, the optimal signal peptide in a specific situation enables a secretion efficiency that balances protein synthesis, folding, and degradation. Because this cannot be rationally calculated, the optimal signal peptide sequence shall be identified using a high-throughput screening method.

Secretory Machinery Components

The Sec and Tat pathways each own specific secretory machinery components for the translocation of secretory proteins across the cytoplasmic membrane.

The Sec pathway. The translocation of secretory proteins using the Sec pathway is conducted in two modes: cotranslational and posttranslational export. During cotranslational export, precursor proteins are translocated during their synthesis by the ribosome. These proteins usually have a highly hydrophobic signal peptide and are mainly membrane proteins (Freudl 2018). The signal peptide of the nascent precursor protein is recognized by the signal recognition particle (SRP) and the complexes of nascent precursor protein and ribosome are transported to the membrane-bound SRP receptor protein FtsY. The SRP particle consists of an RNA molecule (271 nucleotides), an Ffh protein, and two molecules of the Hbsu protein (Akopian et al. 2013). Ffh and FtsY have GTPase activity. These complexes are transported to the Sec translocase for export. The Sec translocase consists of three components: SecA, SecYEG, and SecDF. SecA is an ATPase that acts as a motor for the Sec translocase, and SecYEG and SecDF form an integral membrane transport channel. In the posttranslational export mode, the precursor proteins are synthesized on the ribosome and maintained in a translocation-competent state by intracellular chaperones. Then they are transferred to the membrane translocation channel through intracellular chaperones or soluble SecA. During or immediately after each of the two modes of translocation, the signal peptide is removed by a signal peptidase. *B. subtilis* contains two types of signal peptidases, type I (SipS, SipT, SipU, SipV, and SipW) and type II (LspA), which can cleave the signal peptides of most secretory proteins and lipoproteins, respectively (Tjalsma et al. 1998, Tjalsma & van Dijl 2005). The cleaved signal peptides are subsequently degraded by signal peptide peptidases SppA and TepA (**Figure 1**) (Bolhuis et al. 1999a).

Unlike *E. coli*, *B. subtilis* lacks a SecB component, which has antifolding chaperone activity and escorts secretory substrates from the ribosome to the motor protein SecA. By exchanging the C-terminal 32 amino acids of *B. subtilis* SecA with those of *E. coli* SecA, a hybrid SecA component was constructed that could bind *E. coli* SecB. Co-expression of the hybrid SecA with *E. coli* SecB in *B. subtilis* significantly increased the secretory production of SecB-dependent *E. coli* mutant maltose-binding protein; it also increased the secretory production of SecB-independent *E. coli* alkaline phosphatase (PhoA) by 60% (**Table 1**). These mutation experiments showed that the ability of the SecB–SecA complex to promote secretion is dependent on the SecB targeting function. Furthermore, the C-terminal domain of SecA contains both the SecB binding site and C-terminal linker peptide, which links the SecB binding site with the α-helical scaffold domain. In eubacteria, the SecB binding site of SecA is highly conserved. Perhaps because *B. subtilis* lacks SecB, the C-terminal 22 amino acids of *B. subtilis* SecA, which would normally contain the SecB binding site, are not essential for bacterial viability or protein secretion (Van Wely et al. 2000). Meanwhile, comparison of the SecA amino acid sequences among *B. subtilis* and several other Gram-positive

Table 1 The modifications of host strains for improving recombinant protein production

Host strains	Modifications	Expressed proteins	Secretory expression	Reference
Bacillus subtilis 168	Co-expression of hybrid SecA and *Escherichia coli* SecB	*Escherichia coli* mutant maltose-binding protein; *E. coli* PhoA	Significantly increased; increased 60%	Diao et al. 2012
B. subtilis 168	C-terminal region of SecA deleted	Alkaliphilic *Bacillus* sp. alkaline cellulose; human interferon α	Increased 83%; increased 220%	Kakeshita et al. 2010
B. subtilis KL03	Overexpression of SecYEG	AmyQ	Increased 300%	Mulder et al. 2013
Bacillus megaterium MS941	Overexpression of SipM	*Leuconostoc mesenteroides* dextransucrase DsrS	Significantly increased	Malten et al. 2005
Bacillus licheniformis BL10GS	Overexpression of SppA	AmyL; *B. subtilis natto* nattokinase	Increased 67%; increased 30%	Cai et al. 2017
B. licheniformis ΔOF-3	Overexpression of SipV	*B. subtilis natto* nattokinase	Increased 468%	Cai et al. 2016
B. subtilis 1A751P7	Overexpression of GroE-series chaperones	*B. licheniformis* DSM13 mannan endo-1,4-mannosidase	Increased 125%	Song et al. 2017
B. subtilis IH6622	Overexpression of PrsA	AmyQ	Increased 250%	Kontinen & Sarvas 1993
B. subtilis IH7185	Overexpression of PrsA	*Streptococcus pyogenes* pneumolysin	Increased 150%	Vitikainen et al. 2005
B. subtilis 168	Overexpression of PrsA	*Bacillus anthracis* protective antigen	Increased 250%	Williams et al. 2003
B. subtilis IH6789	Overexpression of PrsA	*B. licheniformis* subtilisin	Increased 200%	Kontinen & Sarvas 1993
B. subtilis BNA	Co-overexpression of PrsA and SecDF	*B. subtilis* 168 lipase LipA	Increased 159%	Ma et al. 2018
B. subtilis 1A237	Co-overexpression of PrsA- and DnaK-series chaperones	AmyL; AmyS	Increased 700%; increased 1,200%	Chen et al. 2015
B. subtilis WB600BHM	Inactivation of *brcA* and overexpression of PrsA	Anti-digoxin single-chain antibody	Increased 250%	Wu et al. 1998
B. subtilis WB800BHM	Inactivation of *brcA* and *wprA* and overexpression of PrsA	Fibrin-specific single-chain antibody fragment	Increased from 0 to 10–15 mg/L	Wu et al. 2002
Lactococcus lactis NZ9000	Overexpression of PrsA	AmyQ	Increased 600%	Lindholm et al. 2006
B. subtilis 168	Deletion of TrxA, introduction of *Staphylococcus carnosus* DsbA and addition of redox-active components	*E. coli* PhoA	Increased 350%	Kouwen et al. 2008
B. subtilis JET2	Inactivation of *dltA*	*B. anthracis* protective antigen	Increased 250%	Thwaite et al. 2002

(*Continued*)

Table 1 (*Continued*)

Host strains	Modifications	Expressed proteins	Secretory expression	Reference
B. subtilis IH6531	Inactivation of *dltB*	Chimeric α-amylase (AmyL, AmyS, and AmyQ)	Increased 200%	Hyyrylainen et al. 2000
B. subtilis IH7375	Inactivation of *dltD*	*S. pyogenes* pneumolysin	Increased 150%	Vitikainen et al. 2005
B. licheniformis IH7896	Inactivation of *dltA*	*Thermoanaerobacter* sp. cyclodextrin glycosyltransferase; *B. licheniformis* penicillinase	Increased 200%; decreased 50%	Craynest et al. 2003
B. subtilis IH8147	Inactivation of *cssR*	AmyL; AmyS; *S. pyogenes* pneumolysin; *Erwinia* spp. pectin methyl esterase	Decreased 50%; decreased 50%; decreased; decreased	Vitikainen et al. 2005

Abbreviations: AmyL, *B. licheniformis* α-amylase; AmyQ, *B. amyloliquefaciens* α-amylase; AmyS, *B. stearothermophilus* α-amylase; PhoA, phosphatase.

and Gram-negative bacteria shows that the C-terminal linker is not well conserved among them. This suggested that the C-terminal domain of *B. subtilis* SecA might be dispensable. Indeed, deletion of the C-terminal 61 amino acids from *B. subtilis* SecA increased the secretory production of alkaliphilic *Bacillus* sp. alkaline cellulose and human interferon α by 83% and 220%, respectively (**Table 1**) (Kakeshita et al. 2010).

Because high-level secretion of recombinant proteins may overwhelm the limited transport capability of the membrane translocation channel composed of SecYEG, the level of SecYEG was increased to optimize the secretory system. Overexpression of SecYEG using an artificial *secYEG* operon increased the secretory production of *B. amyloliquefaciens* α-amylase (AmyQ) by 300% and significantly decreased the accumulation of AmyQ in the cytoplasmic membrane and cytoplasm (**Table 1**) (Mulder et al. 2013). Finally, the overexpression of signal peptidase and signal peptide peptidase in *Bacillus megaterium* or *B. licheniformis* can significantly increase the secretory production of recombinant proteins; therefore, this strategy may also be used to improve secretory production of recombinant proteins in *B. subtilis* (**Table 1**).

The Tat pathway. Although most of the high-level secretion of homologous and heterologous proteins in *B. subtilis* proceeds through the Sec pathway, the Tat pathway also has valuable potential because of its unique translocation mechanism and translocase (Tjalsma et al. 2004). Before translocation through the cytoplasmic membrane, Tat substrates become completely folded in the cytoplasm, with the help of many intracellular chaperones. The folded conformation of secretory proteins can reduce proteolytic degradation, which may be an advantage for the production of easily misfolded proteins (Terpe 2006). In *B. subtilis*, the Tat pathway is composed of three TatA components (TatAc, TatAd, and TatAy) and two TatC components (TatCd and TatCy). TatAd and TatAy form a pore and a docking complex with TatCd and TatCy, respectively, yielding two separate systems: TatAdCd and TatAyCy. The TatAdCd translocase is expressed under phosphate-limiting conditions, whereas TatAyCy translocase is constitutively expressed and can translocate more substrates. TatAc possesses pore-forming activity and can partially complement the functionality of a defective TatAy even though it has no docking activity and cannot, therefore, form an effective translocase with TatCy or TatCd (Goosens et al. 2015). These properties functionally improve the transport capability of the Tat pathway.

Intracellular Chaperones

Three classes of heat-shock genes, which allow the organism to respond to elevated temperatures, have been found in *B. subtilis* (Homuth et al. 1997). The class I heat-shock genes are negatively regulated by a repressor, whereas the class II heat-shock genes are positively regulated by the σ^{32} polypeptide (Haldenwang 1995, Schulz & Schumann 1996). The class III heat-shock genes include *htpG*, *lon*, *ftsH*, *clpP*, and *clpC*; their regulatory mechanism is largely unknown (Deuerling et al. 1995, Kruger et al. 1994, Schulz et al. 1997). The expression of heat-shock genes is also stimulated by the presence of non-native proteins, ethanol, and puromycin. The proteins encoded by these heat-shock genes can ensure the correct folding, oligomerization, and translocation of nascent polypeptides (Georgopoulos 1992). For these reasons, some of these proteins are recognized as molecular chaperones (Ellis & van der Vies 1991). *B. subtilis* contains mainly two types of intracellular chaperone operons: the heptacistronic *dnaK* operon (*hrcA–grpE–dnaK–dnaJ–orf35–orf28–orf50*) and the bicistronic *groE* operon (*groES–groEL*). These class I heat-shock genes are negatively regulated by repressor HrcA through a common regulatory region (**Figure 3**) (Yuan & Wong 1995). This region consists of a σ^{A}-dependent promoter and a 9-bp inverted repeat sequence (CIRCE). The 9-bp inverted repeat sequence is separated by a 9-bp spacer (Zuber & Schumann 1994). Furthermore, the activity of HrcA is regulated by GroE, which indirectly regulates the transcription levels of the *dnaK* and *groE* operons (Mogk et al. 1997). Likely because of their crucial role in mediating the intracellular folding of precursor proteins, the overexpression of the GroE-series chaperones increased β-mannanase expression by 125%, and the overexpression

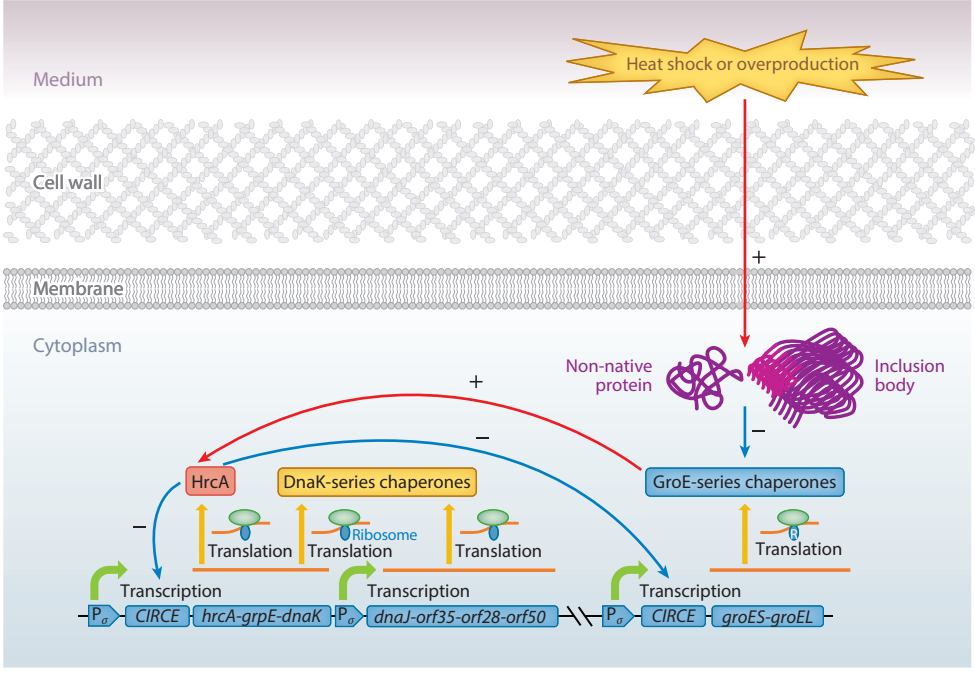

Figure 3

The regulation mechanism of *dnaK* and *groE* operons through repressor HrcA. HrcA that interacts with the CIRCE sequence has a negative regulation effect on the transcription of the σ^{A}-dependent promoter of *dnaK* and *groE* operons.

of DnaK-series chaperones increased the expression of α-amylases AmyL and AmyS by 160% and 173%, respectively (**Table 1**) (Chen et al. 2015, Rashid et al. 1993).

In addition to the DnaK and GroE series of intracellular chaperones, the CsaA protein of *B. subtilis* interacts with precursor proteins and SecA to assist in the export process. Its function is like that of SecB in *E. coli*, which maintains the precursors of secretory proteins in a translocation-competent state (Muller et al. 2000b). In vitro, CsaA can prevent the aggregation of heat-denatured luciferase (Muller et al. 2000a). When interacting with folding variants of its postulated substrate YvaY, the YvaY precursor protein and peptides derived from the YvaY precursor protein, CsaA was found to have a greater affinity to unfolded proteins and positively charged peptides (Linde et al. 2003). However, the precise mechanism of CsaA in the secretory process remains to be fully elucidated.

Extracellular Chaperone Factors

After export from the cytoplasmic membrane, secretory proteins shall quickly fold into the correct conformation to avoid proteolytic degradation. *B. subtilis* own a series of extracellular chaperone factors to assist in the folding of exported proteins, including PrsA, metal cations, thiol-disulfide oxidoreductases, and propeptides.

PrsA. In *B. subtilis*, PrsA is an essential lipoprotein composed of 270 amino acid residues that is anchored to the outer face of the cytoplasmic membrane (Kontinen et al. 1991). It has a parvulin-like domain-sharing sequence similar to the peptidyl-prolyl *cis–trans* isomerases (PPIases) of the parvulin family, is distributed in the cell membrane in a helical pattern, and can form dimers/oligomers (Vitikainen et al. 2004). Strains containing *prsA* mutations are large and spherical, have a thin wall layer in the periphery, and maintain thick cell material in their division septa. In these strains, the levels of penicillin-binding proteins (PBPs) 2a, 2b, 3, and 4 are significantly decreased in a PrsA-dependent manner. The degree of peptidoglycan cross-linking is 2% lower, and the number of pentapeptide side-chains in the cell wall is significantly increased. PBPs, which possess both transglycosylase and transpeptidase activity, are involved in the synthesis of peptidoglycan chains and formation of the murein sacculus (Sauvage et al. 2008). It was speculated that PrsA plays an indispensable role in lateral cell wall synthesis by assisting the folding of PBP2a, PBP2b, PBP3, and PBP4 in the cell wall environment. A membrane proteome analysis of *prsA* mutant strains showed that the level of bacteriophage SPP1 adsorption protein YueB decreased significantly, which suggests that the folding of YueB is assisted by PrsA.

PrsA can also assist the post-translocational folding of exported proteins. PrsA overexpression has been shown to increase the production of several extracellular proteins (**Table 1**). PrsA overexpression increased the expression of the α-amylases AmyQ, AmyL, and AmyS by 2.5-, 4.7-, and 7.1-fold, respectively (Hyyrylainen et al. 2010, Kontinen & Sarvas 1993). PrsA overexpression also increased the homologous expression of lipase LipA by 1.5-fold (Ma et al. 2018). Similarly, PrsA overexpression increased the heterologous expression of *Streptococcus pneumoniae* pneumolysin by 1.5-fold (Vitikainen et al. 2005). These results suggest that higher levels of PrsA may reduce the probability of secretory protein misfolding and quicken their refolding rate, which is expected to reduce extracellular proteolytic degradation and increase the production of exported proteins.

Metal cations. The *B. subtilis* cell wall is composed of peptidoglycan, covalently linked anionic polymers (mainly teichoic acid and teichuronic acid), lipoteichoic acid, and proteins. The first two components account for approximately 40% and 50% of the wall by weight, respectively. Lipoteichoic acids are found mainly in the cytoplasmic membrane, although partially deacylated

forms can also be found in the cell wall (Iwasaki et al. 1986). Anionic polymers and membrane-bound lipoteichoic acids create a high-density negative charge in the cell wall and membrane–wall interface. This negative charge results in the binding of metal cations (e.g., Ca^{2+} and Fe^{3+}) as well as cationic proteins and peptides. Esterification of teichoic acid and lipoteichoic acid with D-alanine, which is regulated by the *dlt* operon (*dltA–dltE*), can decrease the negative charge of the cell wall and membrane–cell wall interface (Hyyrylainen et al. 2000). Inactivation of any of the *dltA–dltD* genes decreases the degree of D-alanylation, yielding an increased degree of negative charge at the cell wall. As the degree of negative charge increases, the cell wall can bind more cationic folding factors, which can act as folding effectors that improve the post-translocational folding of exported proteins (Harwood & Cranenburgh 2008). The density of the negative charge at the cell wall can also modulate the CssRS two-component signaling system, which regulates the expression of quality-control proteases HtrA and HtrB (Hyyrylainen et al. 2007). In a *dltD* mutant strain, secretion stress caused by high-level expression of exported proteins cannot induce HtrA and HtrB expression, causing lower levels of quality-control proteases compared with the wild-type strain. Improved post-translocational folding and decreased quality-control degradation are beneficial to the production of exported proteins. Thus, the production levels of the *Bacillus anthracis* protective antigen, the *S. pneumoniae* pneumolysin, and a chimeric α-amylase consisting of AmyL, AmyS, and AmyQ increased by 250%, 150%, and 200%, respectively, in a *dltA–dltD* mutant strain (Hyyrylainen et al. 2000, Thwaite et al. 2002, Vitikainen et al. 2005).

Although increasing the degree of negative charge at the cell wall and overexpressing chaperone PrsA are each beneficial for secretory protein production, it should be noted that the beneficial effects are restricted to a small percentage of exported proteins (Vitikainen et al. 2005). Also, although increasing the degree of negative charge at the cell wall or overexpressing PrsA increased *S. pneumoniae* pneumolysin production in *B. subtilis*, when these strategies were combined, their effects were not additive.

Thiol-disulfide oxidoreductases. Although many eukaryotic proteins have disulfide bonds, relatively fewer proteins secreted by *B. subtilis* have disulfide bonds. Examples of *B. subtilis* secretory peptides or proteins with disulfide bonds include a bioactive sublancin peptide and the competence-associated protein ComGC (Meima et al. 2002). Because the spontaneous formation of disulfide bonds is slow and nonspecific, bacterial cells contain thiol-disulfide oxidoreductases (TDORs), which catalyze the formation, splitting, and isomerization of disulfide bonds (Tan & Bardwell 2004). The TDORs located in the cytoplasm usually show reductase activity, whereas the TDORs located in the extracytoplasmic space usually show oxidase or isomerase activity (Kouwen et al. 2007, Ritz & Beckwith 2001). In *B. subtilis*, there are at least four TDORs, BdbA, BdbB, BdbC, and BdbD, that show oxidase activity, among which BdbB, BdbC, and BdbD form redox pairs for disulfide bond formation. BdbD is a thiol oxidase present in the extracytoplasmic space that participates in disulfide bond formation and is reduced in the process. BdbD is returned to its oxidized state by transferring electrons to BdbB or BdbC, with BdbC playing a major role in this process (Bolhuis et al. 1999b). BdbB and BdbC, which are paralogous membrane proteins, are returned to the oxidized state by transferring their electrons to quinones in the membrane electron transport chain. BdbD shares sequence similarity with *E. coli* DsbA, whereas BdbB and BdbC share sequence similarity with *E. coli* DsbB (Kouwen et al. 2008). In addition to BdbA, BdbB, BdbC, and BdbD, *B. subtilis* has other TDORs, including cellular reductase TrxA, membrane-embedded reductase CcdA, and CcdA-associated TDORs ResA, StoA, and YneN (Kouwen & van Dijl 2009b). Unlike *E. coli*, *B. subtilis* does not have the two paralogous disulfide isomerases DsbC and DsbG. Instead, disulfide isomerization may be performed by combining reductive (CcdA–ResA) and oxidative (BdbC–BdbD) systems (Kouwen & van Dijl 2009a). Because they help catalyze disulfide

bond formation in ComGC, BdbC and BdbD are required for the development of competence in *B. subtilis*.

Rapid and correct disulfide bond formation is important to avoid proteolytic degradation during the expression of disulfide bond–containing proteins. A variety of strategies that modulate the redox activity of the cellular environment, the cytoplasmic membrane, and the extracytoplasmic environment have been tried to improve disulfide bond formation in *B. subtilis*. Overexpression of the Bdb proteins, singly or combined, has no beneficial effect on the expression of the disulfide bond–containing protein *E. coli* PhoA (Darmon et al. 2006, Kouwen & van Dijl 2009a). However, using a recombinant strategy that included decreasing the level of TrxA, coexpressing *Staphylococcus aureus* DsbA, and adding redox-active compounds to the growth medium increased the expression of *E. coli* PhoA approximately 3.5-fold (Kouwen et al. 2008).

Propeptides. Propeptides, which are present between the signal peptide and the mature protein, have been found in prokaryotic and eukaryotic secretory proteins, including serine protease Bpr, subtilisin E, subtilisin nattokinase, aqualysin I, α-lytic protease, cathepsin L, thermolysin, and carboxypeptidase Y, most of which are proteases (Jia et al. 2010, Meng et al. 2016, Yabuta et al. 2001). Propeptide length varies from 8 to 200 residues. The precursors of these proteins translocate across the cytoplasmic membrane using their signal peptides, and their maturation is assisted by the propeptide. The maturation of these precursor proteins takes place in several stages: folding of the precursor protein, which is assisted by the propeptide; autoprocessing of the precursor protein, during which the peptide bond between the propeptide and mature domain is cleaved; release of the propeptide; and degradation of the propeptide. It has been speculated that propeptides can stabilize the intermediate folding states of precursor proteins, which indirectly accelerates the folding process, through direct interaction of their secondary structures with the mature domain (Gallagher et al. 1995, Wang et al. 1998). Without the propeptide, precursor proteins fold into inactive molten-globule-like intermediates that cannot be secreted across the cell wall (Baker et al. 1992). When their propeptides are provided in *trans*, these intermediates can refold into an active conformation and be secreted into the extracellular space, demonstrating that the propeptide does not need to be covalently attached to assist precursor protein folding. The synchronized folding and autoprocessing of precursor proteins are relatively rapid processes; release of the active mature protein is the rate-determining step. Compared with the autoprocessed precursor protein, non-native folding intermediates and precursor proteins that have not been autoprocessed are proteolytically unstable. For the maturation of protease precursors, the rate-limiting step in the release of an active mature protease reduces the degradation of the precursor proteins that have not been autoprocessed. After the release of the first active mature protease, the propeptide domains of other autoprocessed precursor proteins are degraded in *trans*, triggering an exponential cascade activation process. With the degradation of the propeptide, the mature proteins are locked into a stable conformation.

When *B. subtilis* was cultivated in L-broth, its α-amylase AmyE was present during the log phase in a form that lacked its N-terminal 33-amino-acid signal peptide, whereas during the stationary phase it was present in a mature form that lacked the 41 N-terminal amino acids. The short sequence removed after removal of the signal peptide was probably the propeptide of AmyE, even though it was shown to be dispensable for the secretion, folding, and stability of AmyE (Takase et al. 1988). When human interferon α, which is sensitive to proteolysis, was coexpressed with the propeptide of AmyE in a protease-proficient strain, it was detected in the culture medium, demonstrating that the AmyE propeptide can enhance the secretion of heterologous proteins (Kakeshita et al. 2010). The exact mechanism by which AmyE propeptide enhances the secretion of heterologous proteins remains to be elucidated.

PROTEOLYTIC DEGRADATION OF NATIVE AND RECOMBINANT PROTEINS

Physiological Functions and Characteristics of Membrane and Extracellular Proteases

B. subtilis can secrete many proteins into the cytoplasmic membrane, membrane–cell wall interface, cell wall, and culture medium. The membrane proteases (HtrA and HtrB) and extracellular proteases (NprE, AprE, NprB, Bpr, Mpr, Epr, Vpr, and WprA) of *B. subtilis* have a series of important physiological functions related to protein degradation (**Figure 1**). HtrA and HtrB control the quality of the membrane proteins and secreted proteins by degrading misfolded membrane proteins. They help the folding of at least 15 secreted proteins (BglC, CotN, LipB, MsmE, SacC, YwmC, YbbC, YraI, YjdB, YdaJ, YvfO, YqxI, YdhT, YwoF, and YxiA) (Krishnappa et al. 2013). HtrA and HtrB can also cleave lipid-modified N-terminal cysteine residues, with or without additional residues of the mature protein, from lipoproteins Deppe, YurO, YtcQ, and YxiP, which releases the proteins from the membrane surface into the growth medium. Therefore, HtrA and HtrB are important for maintaining cell integrity. Native proteins can also be degraded by extracellular proteases other than HtrA and HtrB. Proteomic analysis revealed at least 43 kinds of native proteins, including membrane proteins (YueB, PbpB, LtaS2, YqgS, OrfRM1, YabE, YwbM, YkoJ, FtsH, YacD, YpuA, RsiX, AnsA, QoxA, YttA, YpjP, YoeB, YpmB, AtpF, YdjM, YqzC, YqgA, FtsL, and DivIC), lipoproteins (AppA, PrsA, RbsB, YcdA, YjhA, and YvrC), and secreted proteins (AspB, GtaB, GroS, YwsB, YddT, YomL, YheN, YfhK, YqxI, YvgO, YjcM, YwaD, and YocH), that are potential substrates of the eight extracellular proteases. In addition, proteases WprA and Epr can degrade the wall-bound autolysins LytE and LytF, and one of the eight extracellular proteases can degrade the autolysin LytD (Yamamoto et al. 2003).

There is no consensus concerning whether PrsA can be degraded by HtrA and HtrB. The level of PrsA in the medium of strain BRB14, which is deficient in eight extracellular proteases as well as HtrA and HtrB, was higher than that found in the medium of strain BRB08, which is deficient only in eight extracellular proteases, whereas the level of PrsA in the cell envelope of BRB14 was comparable with that of BRB08 (Krishnappa et al. 2014). Proteomic analysis revealed that protease WprA is involved in the cleaving of PrsA from the membrane and that secreted PrsA can be degraded by one or more of the other seven extracellular proteases. Like PrsA, HtrA and HtrB can be degraded by WprA and other extracellular proteases. Although WprA can degrade HtrA and HtrB, WprA is required for the expression of *htrA* and *htrB* or the stability of HtrA and HtrB when other extracellular proteases are present, suggesting a chaperone-like activity for WprA. Furthermore, using ectopic expression of an extracellular protease in an extracellular protease mutant strain that is deficient of all extracellular proteases except WprA, proteases Bpr and NprE were found to be involved in the degradation of HtrA, HtrB, and PrsA.

HtrA and HtrB possess similar functions in controlling the quality of secretory proteins and are regulated by the CssR and CssS two-component system. The levels of HtrA and HtrB in protease-deficient strains show complementary features. During an extracellular proteomic analysis of protease-deficient strains BRB11, which is deficient in *htrA*, and BRB12, which is deficient in *htrB*, the level of HtrA was elevated in BRB12, whereas no HtrB was detected in BRB11. This suggested that HtrB cannot easily be cleaved from the cytoplasmic membrane or that the HtrB can easily be degraded by extracellular proteases (Pohl et al. 2013). The latter speculation was verified by another proteomic analysis as follows: In the culture media of a series of seven extracellular protease-deficient strains that sequentially lacked an increasing number of proteases, the levels of HtrB increased sequentially, whereas the levels of HtrA remained essentially the same, demonstrating that HtrB is more sensitive to proteolysis than HtrA.

Optimization of Extracellular Proteolytic Activity for Better Recombinant Protein Production

Heterologous proteins are more sensitive to proteolytic degradation than native proteins because they fold slower after membrane translocation and have a higher propensity to misfold (Westers et al. 2006a,b). The proteolytic degradation of heterologous proteins can be reduced using two strategies: improving the secretory protein-folding environment and reducing extracellular proteolytic activity. To pursue the latter strategy, protease-deficient strains have been constructed, among which *B. subtilis* WB600, WB700, and WB800 (constructed from *B. subtilis* 168) are the most widely used for protein recombinant expression. In protease-deficient strains, the degradation of incompletely synthesized, misfolded, and properly folded recombinant proteins is greatly reduced. Meanwhile, as described above, the levels of PrsA, HtrA, and HtrB are higher in protease-deficient strains. These proteins may promote correct folding and ensure quality control of recombinant proteins. Thus, the secretion of most extracellular proteins, especially proteolytically sensitive proteins, is increased in these protease-deficient strains (Lee et al. 2000, Murashima et al. 2002).

Because WB800 has lower extracellular proteolytic activity than WB600, WB800 usually secretes higher levels of recombinant protein than WB600. However, the levels of *Bacillus naganoensis* pullulanase and *Clostridium thermocellum* endoglucanase secreted by WB600 were higher than those secreted by WB800 (Liu et al. 2012, Song et al. 2016). Production of these recombinant proteins in WB600 may have been increased because of increased cell growth. Compared with WB800, the heterologous recombinants of WB600 usually exhibit better growth with lower cell lysis, which is related to the degradation of misfolded proteins and autolysins by the remaining extracellular proteases WprA and Vpr (Yamamoto et al. 2003). In another example, *Bacillus deramificans* pullulanase, an easily misfolded protein, was expressed in strains with different degrees of protease deficiency (Zhang et al. 2018). When cultivated in a three-liter fermenter, the extracellular pullulanase activity and dry cell weight of a recombinant strain retaining extracellular proteases WprA and Vpr (2,449.6 U/mL and 67.0 g/L) were 2.34- and 1.19-fold greater, respectively, than those of a recombinant strain deficient in all eight extracellular proteases (1,047.6 U/mL and 56.1 g/L). Purification and kinetic parameter analysis of four different three-liter fermenter cultivations revealed that the percentage of misfolded pullulanase increased as extracellular proteolytic activity decreased. Because *B. subtilis* has a limited capacity for the expression and secretion of heterologous proteins, the large percentage of misfolded forms decreases the expression of active pullulanase. In this situation, a suitable level of extracellular proteases, especially WprA, plays an important role in quality control and contributes to the increase in extracellular pullulanase activity.

To further reduce the proteolytic activity of *B. subtilis* strains, the effect of HtrA and HtrB deficiencies on the production of heterologous proteins was investigated. Because HtrA and HtrB expression are strictly regulated by the CssRS two-component system, an HtrA and HtrB deficiency can be created by disrupting *cssR* or *cssS* (Hyyrylainen et al. 2005). Compared with the wild-type strain, the expression levels of AmyS, AmyL, and *S. pneumoniae* pneumolysin were decreased in a *cssR* mutant strain (Vitikainen et al. 2005). During the expression of heterologous proteins, the *cssR* or *cssS* mutant strains show a slower growth rate compared to that of their wild-type counterpart, which may be caused by the accumulation of misfolded proteins (Hyyrylainen et al. 2001). Meanwhile, an HtrA deficiency, an HtrB deficiency, or deficiencies of both HtrA and HtrB all show a negative effect on the extracellular production of *B. anthracis* recombinant protective antigen (rPA). It was speculated that both HtrA and HtrB can degrade misfolded or unfolded rPA, whereas their existence is crucial for the expression of rPA (Pohl et al. 2013).

Proteome analysis suggested the existence of other, unidentified extracytoplasmic proteases, as proteolytic activity can be found in a strain that is deficient in two membrane proteases and eight extracellular proteases (Krishnappa et al. 2013). Along with the fact that the membrane proteases HtrA and HtrB are important for bacterial viability, which cannot be inactivated, the proteolytic activity that remained in the strains deficient in seven or eight extracellular proteases was very low, demonstrating that the potential to further reduce extracellular proteolytic activity is limited. Meanwhile, the ideal extracellular proteolytic activities of the host strains used to produce different recombinant proteins are different, which may be related to the negative effect of protease deficiency on cell growth and the sensitivity of recombinant proteins to proteolysis. Secretory production of recombinant proteins may be further increased by optimizing the proteolytic activity of the host strain.

Modification of Cell Lysis Degree in a Protease-Deficient Strain for Better Recombinant Protein Production

Peptidoglycan, a crucial cell wall component, has a dynamic structure in which its synthesis and degradation are balanced. This balance is required for cell proliferation. Peptidoglycan hydrolase, also called autolysin, participates in peptidoglycan disassembly by cleaving the covalent bonds in peptidoglycan. *B. subtilis* has at least 35 genes that encode autolysins involved in regulating cell separation, motility, sporulation, competence, and lysis (Smith et al. 2000, Vollmer et al. 2010). When classified according to their cleaving sites, these autolysins are divided into four kinds: muramidases, amidases, endopeptidases, and glucosaminidases (Smith et al. 1996). In *B. subtilis*, autolysins LytC, LytD, LytE, LytF, and LytG are produced during vegetative growth (Blackman et al. 1998). LytD and LytF are tightly regulated by RNA polymerase subunit σ^D, and 70% of LytC expression is controlled by σ^D (Lazarevic et al. 1992, Margot et al. 1999). LytC and LytD are the major autolysins produced during vegetative growth; LytC is important for flagellar function but is not involved in the cell-separation process (Chen et al. 2009). LytE and LytF, which are involved in cell separation, have three and five lysM domains, respectively, within their N-terminal regions. LytG is a 32-kDa exoglucosaminidase important for peptidoglycan structure determination. Strains containing a single mutation in *lytF*, *lytE*, or σ^D form filamentious or fiber cells, whereas strains containing a single mutation in *lytC*, *lytD*, or *lytG* form normal morphological cells. Disruption of *lytE* and *cwlO*, which encode two endopeptidases involved in cleaving D-γ-glutamyl-*meso*-diaminopimelic acid linkages in peptidoglycan, can seriously affect cell elongation and shows synthetic lethality for cell proliferation (Bisicchia et al. 2007, Hashimoto et al. 2012).

Proteases play a crucial role by cleaving misfolded proteins present at the membrane–cell wall interface. This avoids blockage of the secretory pathway, which is important for the normal secretion of proteins. Proteases also degrade wall-bound autolysins, decreasing the level of autolysins (Tjalsma et al. 2004). Compared with a protease-proficient strain, protease-deficient strains secrete a higher level of cannibalism factor SdpC, which can kill the nonsporulating cell, and a lower level of protein SivC, which can repress the initiation of sporulation (Pohl et al. 2013). Consequently, the degree of cell lysis is increased in protease-deficient strains, and the major extracellular proteases AprE and NprE account for a larger part of this effect than the extracellular proteases NprB, Bpr, Mpr, and Epr (Stephenson et al. 2010, Westers et al. 2004). The greater degree of cell lysis in protease-deficient strains is detrimental for protein production because intracellular proteases released during lysis can degrade extracellular proteins and reduce the amount of time during which production is efficient. Decreasing the degree of cell lysis may be a good strategy to improve protein production in protease-deficient strains because it compensates for the negative

effects of protease deficiencies on cell growth (**Figure 2**). The appropriate disruption of autolysin genes has been shown to improve recombinant protein production in protease-proficient strains. A *lytC* deficiency in *B. subtilis* ATCC 6051 increased cell density, prolonged the exponential and transient growth phases, and increased AmyE secretion (Kabisch et al. 2013). The inactivation of *sigD*, *lytE*, and *lytD* in *B. subtilis* Δ*upp* yielded a long, filamentous strain with lower autolysin content. The strain, which shows advantageous characteristics, including better growth, a greater sensitivity to antibiotics, greater ease of precipitation, and higher tolerance to salt in the medium, secreted 1.2-fold more AmyE (Zhao et al. 2018). Furthermore, the mutant strain obtained by removing *lytC*, prophage gene *xpf*, and cannibalism factor genes *skfA* and *sdpC* from the genome of *B. subtilis* 168 showed higher cell density and secreted 1.72-fold more *E. coli* β-galactosidase and 2.6-fold more *B. subtilis* (*natto*) nattokinase (Wang et al. 2014).

CONCLUSION

B. subtilis, widely used as a microbial cell factory in industrial production, has been thoroughly studied. This has led to a basic understanding of those aspects leading to efficient protein production and has provided abundant information for improving its protein production capability. Application of the CRISPR–Cas9 system of genome editing and transcriptional regulation makes strain modification much more convenient. The CRISPR–Cas9 system is also a powerful tool for the identification and analysis of genes essential for efficient protein production. In addition to advances in gene-editing methods, a more complete understanding of the genomic heritage, provenance, and phenotype of legacy strains will promote genotype editing of existing engineered strains and the modification of undomesticated strains to provide better expression hosts. With deeper knowledge of the mechanisms of protein secretion and folding, many effective strategies were employed to improve the secretion of recombinant proteins, including modifying, overexpressing, coexpressing, and disrupting the factors involved. Finally, proteomic analysis has shown that the degradation of native proteins by extracellular proteases is important for cell integrity, and their degradation of recombinant proteins affects protein production. Optimizing the extracellular proteolytic activity of the host strain maintains a balance between these two aspects, and reduction of the autolysin content of protease-deficient strains can improve recombinant protein production. Overall, with the development of genome-editing methods, engineering of undomesticated strains, and modification of protein expression assisted factors, the recombinant protein production by *B. subtilis* will be further improved.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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