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Review

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Improving Protein Production on the Level of Regulation of both Expression and Secretion Pathways in *Bacillus subtilis*

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The well-characterized gram-positive bacterium *Bacillus subtilis* is an outstanding industrial candidate for protein expression owing to its single membrane and high capacity of secretion, simplifying the downstream processing of secretory proteins. During the last few years, there has been continuous progress in the illustration of secretion mechanisms and application of this robust host in various fields of life science, such as enzyme production, feed additives, and food and pharmaceutical industries. Here, we review the developments of *Bacillus subtilis* as a highly promising expression system illuminating strong chemical- and temperature-inducible and other types of promoters, strategies for ribosome-binding-site utilization, and the novel approach of signal peptide selection. Furthermore, we outline the main steps of the Sec pathway and the relevant elements as well as their interactions. In addition, we introduce the latest discoveries of Tat-related complex structures and functions and the countless applications of this full-folded protein secretion pathway. This review also lists some of the current understandings of ATP-binding cassette transporters. According to the extensive knowledge on the genetic modification strategies and molecular biology of *Bacillus subtilis*, we propose some suggestions and strategies for improving the yield of intended productions. We expect this to promote striking future developments in the optimization and application of this bacterium.

Keywords: Promoter, ribosome-binding site, signal peptide, Sec pathway, Tat pathway, ATP-binding cassette transporter

Introduction

The gram-positive bacterium *Bacillus subtilis* is by far one of the most widely used hosts for the mass production of recombinant proteins in biotechnology. The popularity of this remarkable microorganism, in both basic research and practical applications of secreting target proteins, is based on its characteristics, such as its status of being generally recognized as safe (GRAS), being sequenced totally, ease of manipulation, absence of significant codon bias, and genetically well-characterized expression systems. Furthermore, its production has widely been used in fields of red and white biotechnology, such as the pharmaceutical, food, and feed industries [21, 23, 64, 115, 132].

In general, *Bacillus subtilis* is much more suitable for the production of secretory recombinant proteins than *Escherichia coli*. Lacking an outer membrane, *B. subtilis* easily targets proteins of interest to the culture medium, simplifying downstream processing and eliminating the need of cell lysis and chemical process techniques for the isolation and purification of overexpressed products. Well-characterized genetics and the availability of an increasing number of cloning vectors and mutant host strains are provided by the *Bacillus* Genetic Stock Centre (BGSC) [99]. Being a long journey for the cargo proteins from birth to maturity, the steps from transcription to secretion include a variety of unknown bottlenecks for the final production [61, 63, 79, 90].

During several decades, we have witnessed the advancements of different industrial workhorses in aspects of genetic engineering and secretory machine mechanisms. Shedding light upon the latest improvements of this expression system, this review concentrates on current understandings of the Sec and Tat pathways, transformation methods, and genetic engineering strategies. This review will drive developments of *Bacillus subtilis* as a high-performance candidate in the production of secretory recombinant proteins.

Expression System

To improve the protein production, regulation can take place at the levels of both transcription and translation. Up to now, the basic economical and effective approach still remains the optimization of promoters at the level of transcription. Therefore, rational choice of the adequate promoter system for a specific protein of interest remains important. However, selecting suitable ribosome-binding sites (RBS) and signal peptides (SP) has also led to high yields of protein production, but at the level of translation.

Promoter

Regulation of gene expression in *Bacillus subtilis* primarily occurs during transcription, in which RNA polymerase (RNAP) binds to the corresponding promoter sequences. This RNAP consists of the core enzyme (α, α′, β, β′) and the σ-factor, which determines the specifity of RNAPs. The σ-factors of vegetative cells include σ^A, σ^B, σ^C, σ^D, σ^H, σ^L and σ^E, σ^F, σ^G, σ^K, where the latter group belongs to the sporulation-specific σ-factors [30]. In addition, the use of pro-protein sequences and anti-σ-factor proteins, in order to modulate σ-factor activity, indicates that proteins are expressed under sophisticated conditions of transcriptional control.

Promoter regions share several characteristics [32]: the consensus -35 sequence of TTGACA, a -10 sequence of TATAAT, and a spacer between the -35 and -10 regions usually comprising 17 base pairs, with some comprising 18 base pairs [73]. A -15 region (or TGn motif) from position -17 to -14 contains TRTG (where R stands for A or G). purine initiation principle means that the translational initiation site usually includes A or G. Most promoters having 4–7 base pairs between the -10 and +1 sites usually are enriched by AT pairs. Another AT-rich region upstream from the -35 region and designated as UP element also counts as important in the efficient application of *Bacillus subtilis* promoters. It can be considered as a transcription-enhancing region interacting with the C-terminal domain (CTD) of the α-subunit of the RNA polymerase [1].

It was demonstrated that enrichment of AT in the natural UP element of P_{groE} leads to a 2-fold increased activity of the report gene *bgaB* [86]. There is distant genetic controlling in the *yqiHIK* promoter [24]: The activity and osmotic regulation of the *yqiHIK* promoter are also influenced by a 53 bp sequence comprising an AT-rich DNA segment, positioned 180 bp upstream of the -35 sequence. When changing the core region of PgroE into a consensus -35 and -10 sequence, the promoter activity increases about 9-fold and 16-fold, respectively, and 100-fold when both changes are combined in one single promoter. Point mutations within the -15 region also result in significantly increased activity. After combining the optimized sequences of the UP element and the -35, -15, and -10 regions, intracellular product accumulation of the reporter gene *bgaB* increases from 9% to 30% of the total cellular wild-type protein amount. Thus on one hand, it is useful to link all these optimized elements in one single promoter. On the other hand, one needs to deal with the degradation of overexpressed recombinant proteins by knocking out protease genes, since efficient transcription does not guarantee a concomitant increase in translation and correctly folded state of proteins.

During the past few years and according to these major elements, there have been many reports about strong promoters, their corresponding expression systems, and other types of promoters in order to facilitate the experimental and industrial utilization. Here, we summarize as follows (Table 1).

Apart from searching for suitable promoters for the expression of specific proteins in published literature or patents, promoter screening *via* the pHT06 vector allows the cloning and analysis of strong promoters in *Bacillus subtilis* on X-Gal plates and IPTG added in appropriate concentrations [85].

Ribosome-Binding Site

The RBS element plays an important role in the translational efficiency of *Bacillus subtilis* even though the factors determining mRNA stability are far from being completely identified and understood [6]. It was demonstrated that the important elements involved are the Shine-Dalgarno (SD) sequence, known to form Watson-Crick base pairs with the 16S rRNA of the 30S subunit, the spacer length between the SD sequence and initiation codon, the initiation codon itself and the spatial organization of the initiation domain of the mRNA [108].

It has been reported that a strong optimal RBS appears to

Table 1. Different types of promoters and corresponding expression systems of *Bacillus subtilis*.

Type	Promoter	Vector	Description	Reference
Strong promoter	P_{yxiE}	pYG123	β-Gal production directed by P*yxiE* was far higher than that by P_{43}	Zhang *et al.* [126]
	$P_{glv\text{-}M1}$	pJRINM1	High expression strength and alleviates the repression caused by glucose	Ming *et al.* [72]
	P_{aprN_M10}	pHY10	Relative activity of nattokinase is 236% in *Bacillus subtilis* DB104	Wu *et al.* [118]
	P_{lapS}	pLus-Hyb	Strong constitutive promoter	Yang *et al.* [123]
Inducible promoter	$P_{grac\ 100}$	pHT100	IPTG-inducible strong promoter	Phan *et al.* [86]
	P_{mtlA}	pKAM12	Mannitol-inducible promoter	Heravi *et al.* [33]
	$P_{glv\text{-}M1}$	pJRINM1	Maltose-inducible promoter and alleviates the repression caused by glucose	Ming *et al.* [72]
	P_{T7}	pMT1	IPTG	Chen *et al.* [15]
	P_{xylA} P_{xylR}	pSWEET-bgaB	Xylose-dependent system	Bhavsar *et al.* [8]
	SacB promoter	pMA5	Sucrose-inducible	Liu *et al.* [66]
	P*gcv*_riboswitch and signal sequence	pT33	Glycine tandem riboswitch	Phan *et al.* [88]
Others	Modified P_{cry3Aa}	pD82	Auto-inducible: stationary phase-specific auto-inducible	Lee *et al.* [58]
	P_{manP}	pMW168.1	Auto-inducible; vectors relatively stable	Wenzel *et al.* [114]
	P_2	pHybgaB2	Temperature-inducible promoters: higher production at 45°C than at 37°C	Li *et al.* [60]
	Des promoter	pAL10 pAL12	Cold-shock inducible: significantly more enzyme present at 25°C as compared with 37°C	Thuy and Schumann [101]
	GsiB promoter	pLDV2	Heat-shock stress-inducible and glucose starvation-inducible (*gsiB*) gene promoter	Paccez *et al.* [82]
	P_{23S}	pOMH	Osmolarity-inducible promoter	Zhang *et al.* [129]
	P_{LapS}	pLus-Hyb	Double promoter	Yang *et al.* [123]
	BJ27UP promoter	pBAP1 pBAP2 pBAP3	Constructed from strong promoter BJ27Δ88 and a fragment of the tac promoter Hybrid promoter	Kim *et al.* [49]
	HpaII+amyR2	pHA-TSαGT	Dual promoter a second constitutive strong promoter downstream of the HpaII promoter, high-level overexpression of TSαGT gene	Kang *et al.* [46]

be crucial for increased stability of the σ^B-dependent general stress protein (gsiB) mRNA [43]. This is consistent with the observations showing that the mRNA half-life is rapidly reduced after removing a stem-loop structure at the 5′ end or diminishing the strength of the RBS [31]. Furthermore, inactivation of the RBS or the start codon results in a 4-fold decrease of the *ermC* mRNA half-life. This plasmid-borne erythromycin resistance (*Emr*) gene is a 254-nucleotide model mRNA used to study the effects of translational signals and ribosome transit on mRNA decay in *Bacillus subtilis* [97]. Additionally, the sequence upstream of the translational start codon is crucial for the long half-life of the *gsiB* mRNA rather than the translated sequence, which is revealed by the northern blotting experiments with different *gsiB-lacZ* fusions.

To increase mRNA stability in order to elevate the production of the target protein, Phan *et al*. [87] proposed a 5′-mRNA controlled stabilizing element (CoSE) by combining a transcriptional operator *lacO* stem-loop with a strong RBS_{gsiB}, fused to the promoter *groE*. With further suitable spacer in between, the mRNA stability presented a half-life of more than 60 min. This indicates that this method is a

promising tool for enhancing protein expression levels.

Moreover, for artificial elements, tools for automated design of synthetic RBSs have been developed to control protein expression [93, 94, 112]. In their statistical thermodynamic equilibrium model, the translation initiation rate is quantified according to the strength of the molecular interactions between an mRNA transcript and the 30S ribosome complex. The attack of 5′ endoribonucleases can be prevented when the initiating ribosomes mask the nuclease recognition sites near the RBS; thus, mRNA stability is tightly coupled to the translation initiation frequency [14]. The fact that the spacer length between a SD sequence and the start codon influences the translational efficiency, combined with the statistical thermodynamic equilibrium model, led to the development of the software RBSDesigner. This software roughly enables to define yield of protein expression by specific RBS design [77].

Signal Peptide

Proteins destined to be transferred across the cytoplasmic membrane possess N-terminal signal peptides, which contain three distinct regions: a positively charged N-terminal domain, followed by a hydrophobic core region, the so-called H-region, and the C-terminal region with a signal peptidase cleavage site [90] (Fig. 1). Signal peptides consist of short stretches of amino acids and will be immediately removed and degraded by specialized signal peptidases and signal peptide peptidases (SPPases) after the protein delivery to the correct destination. During translocation, the positively charged N-terminal domain interacts with both the translocation machinery and negatively charged phospholipids in the lipid bilayer of the membrane. Helix-breaking residues of glycine or proline, which are frequently present in the middle of H-regions, allow the signal peptide to form a hairpin-like structure that can insert into the membrane.

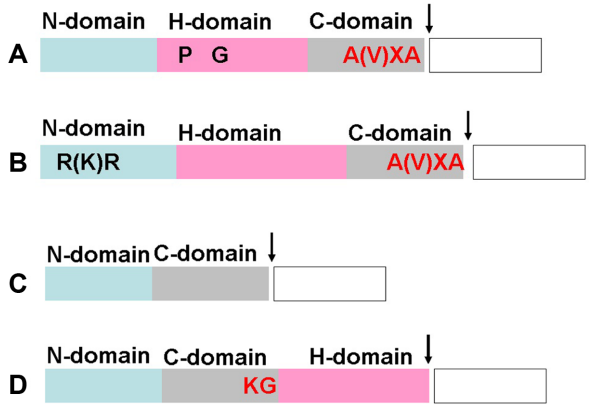

Fig. 1. Scheme of the signal peptides of different secreting pathways of *Bacillus subtilis*.
(**A**), (**B**), (**C**), and (**D**) represents the Sec-dependent signal peptide, the Tat-dependent signal peptide, the ABC-dependent signal peptide, and the Com-dependent signal peptide, respectively. The arrowhead points to the cleavage site.

Respectively, signal peptides can be divided into two types, depending on the signal peptidases that are responsible for the release of mature proteins to final positions by cleavage of signal peptides, as described by Pohl and Harwood [90].

Nevertheless, signal peptides can also be assigned to other classes, according to export pathways and signal peptidase cleavage sites, as summarized by Tjalsma *et al.* [102, 103] and Ling *et al.* [63]. Both papers report of Sec-type signal peptides and twin-arginine signal peptides. The main difference is the type of SP according to their signal peptidase. The lantibiotic and pheromone signal peptides consist of only N- and C-terminal domains, but lack the H-domain, which is removed by specific ABC transporters.

Furthermore, the Com-dependent pseudolipin-like signal peptides, detected at four proteins of *Bacillus subtilis*, are designated as ComGC/GD/GE and GG. Owning 33 residues on average, its consensus sequence (K-G-F) is located between the N and H-domains. Bypassing Sec and Tat pathways by its own specific pathway, pseudolipin-like signal peptidases work at the cytoplasmatic side of the membrane. By this, they take part in aminomethylation and DNA-binding processes to the membrane during transformation [16, 102].

The other type of signal peptide is followed by a propeptide. In this case, the signal peptide is removed by a Type I enzyme and the propeptide is removed either autocatalytically or by a coexisting protease.

As the hydrophobicity of the signal peptide is a critical factor for early stage protein secretion [125], researchers have developed the signal peptide prediction tool SignalP, which helps choosing signal peptides. It treats signal peptides with a calculated high D-score (usually above 0.5) as the suitable ones to enhance the production. Unfortunately, the D-score and its real secretion amounts do not seem to correlate, as was determined by a complex pattern of events. In order to construct a powerful strategy in optimizing the secretion of heterologous proteins, Brockmeier *et al.* [11] reported a SP library to screen the individually optimal signal peptides for each secreted heterologous protein, which was achieved by a high-throughput screening method. Cutinase, an enzyme from *Fusarium solani pisi* and the metagenomic cytoplasmatic esterase, which acts as a model

reporter for naturally unsecreted proteins, were chosen as two lipolytic enzyme reporters of heterologous protein secretion. A total of 148 of the 173 SP-cutinase fusions could be expressed in *Bacillus subtilis,* with different lipolytic activity of the culture supernatants. Seven SPs with strong, moderate, and low efficiencies were further analyzed by western blotting and pulse-chase experiments. The results indicate that translocation and SP processing efficiencies are not the sole determinants responsible for protein secretion. When the most efficient SP for cutinase is fused to the esterase, the secretion level only amounts to about 5% of the most efficient SP for esterase. This phenomenon underlines the secretion target specificity of the best SP, as the interaction between the SP and the mature protein is influenced by the composition of the amino acids of the two fragments.

Consistent results had been reported by Degering *et al*. [18], who constructed a signal peptide library containing 173 predicted SPs originating from *Bacillus subtilis* and 220 from the *Bacillus licheniformis*. They identified two SPs resulting in a 7-fold increase of subtilisin activity in the medium supernatant, compared with wild-type SP. These two SPs are not from high secreted enzymes like AprE and NprE. This indicates that SPs from highly secreted proteins do not necessarily lead to efficient secretion of proteins. They also recommend using *Bacillus subtilis* for preliminary high-throughput screenings of SP libraries of specific proteins. After this, one can transfer the best-performing SP to a production strain, in case the production strain, such as *Bacillus licheniformis*, is not amenable to molecular manipulations, including DNA transformation.

Another alternative for increasing cutinase secretion by finding the beneficial amino acids of the SP in *Bacillus subtilis* is saturation mutagenesis of the positively charged N-domain (positions 2–7) [12]. This method enables the identification of single point mutants leading to 2- to 4-fold increased amounts of cutinase expression. Not only is the amount of cutinase in the supernatant improved, but also the quality of the protein with respect to its specific activity, even though a reduction of the net charge of the SP from +3 to +2 is observed, which usually is associated with a less effective interaction of the SP with its binding partners [84].

Owing to our limited knowledge about the secretion mechanism, it is still difficult to significantly improve protein secretion by rational design of amino acids. Both the construction of SP libraries and directed evolution, and their combination, seem to be efficient approaches. However, it should be noted that an appropriate high-throughput screening method for specific proteins is necessary at the same time.

Process of Secretion Pathways and Related Proteins

Proteins are transported to their destinations after synthesis through distinct mechanisms [115] (Fig. 2). *Bacillus subtilis* has evolved several pathways to fulfill this request in order to adapt to the changing environment: the general protein secretion pathway (Sec), the two-arginine pathway (Tat), and the ATP-binding cassette (ABC) transporters. Here, we mainly introduce the detail of the Sec pathway and the relevant proteins in this channel. We also focus on the latest discoveries about the Tat pathway. The ABC transporters are simply described.

Sec Pathway

Recognition and targeting. Immediately after the synthesis of precursor proteins, the cytoplasmic chaperones guarantee that preproteins gather the state of translocationcompetence before their signal peptides are recognized by the signal recognition particle (SRP) and are combined with the SRP receptor FtsY [2, 127].

The highly conserved ribonucleoprotein complex SRP consists of a scRNA, two histone-like proteins (HBsu), and Ffh, which is a 446-amino-acid protein interacting with scRNA and forming a stable complex. The SRP RNA (long SRP RNA) of *Bacillus subtilis* contains an Alu domain, which arrests protein biosynthesis by competing with elongation factors that bind on ribosomes. Retarding translation gives SRP time to interact with its membrane receptor FtsY. The DNA-binding protein HU1, a part of HBsu, has been suggested to be part of the bacterial Alu domain [48].

Both Ffh and FtsY belong to the widely conserved family of SRP-GTPase. The GTP hydrolysis at FtsY and SRP affects the release of the ribosome-nascent chain complex (RNC), the complex comprising the nascent peptide chain, tRNA and the ribosome, from SRP to the Sec translocation channel [116]. However, a third SRP-GTPase, named FlhF (flagellum-associated protein), seems to be dispensable for protein secretion. It has been observed that there is no significant difference of the transcriptional level of flhF between normal protein secretion and high-level expression of the α-amylase AmyQ from *Bacillus amyloliquefaciens*. *flhF* and its neighboring genes, such as *yxlH*, are involved in cell motility. *flhF* has a minor role, as the single mutation in *flhF* does not affect cell motility whereas the combined *flhF* and *ylxH* mutations affect motility more severely than a

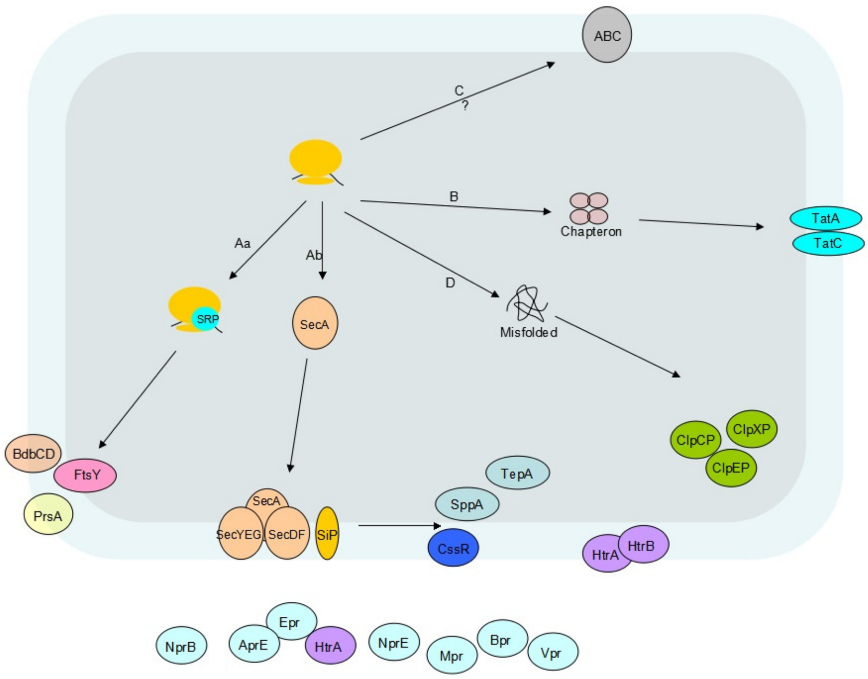

Fig. 2. Scheme of the different secreting pathways of *Bacillus subtilis*.
(**A**) The Sec pathway: the signal recognition particle (SRP) recognizes the signal peptides of the ribosome-nascent chain complexes, and the cytoplasmic chaperones guarantee the translocation-competent state. Then pre-proteins combine with the SRP receptor FtsY (Aa), or translocate *via* the SecYEG translocase complex (Ab). Then the signal peptide is cut and degraded by the Spases, SppA and TepA, respectively, and finally modified and checked by other complexes, such as BdbCD and HtrAB. (**B**) The Tat pathway: pre-proteins fold in the cytoplasm with the help of cofactors, and then pass through the Tat translocase under the presence of the pH gradient across the cytosolic membrane. After translocation, type I Spases cut down the signal peptides and the remaining proteins secrete into the medium. (**C**) The ABC pathway: proteins secrete through the ATP-binding cassette, by mechanisms that have not been clearly understood yet. (**D**) Misfolded proteins are degraded by ClpCP, ClpXP, and ClpEP in the cytoplasm or by extracytoplasmatic proteases such as NprB, AprE, Epr, and/or other proteases.

single *ylxH* mutation. In addition, the function of FlhF can be replaced by FtsY but not *vice versa* [124]. The preliminary X-ray crystallographic analysis of FlhF fragments described its possible importance for the assembly of polar flagella but not in protein secretion [5].

Transferring and passing. Second, the translocation of proteins across the membrane is performed through the channel of the SecYEG translocase complex and the accessory protein SecDF. At the same time, SecA catalyzes the hydrolysis of ATP, providing energy for crossing the membrane.

The function of SecYEG in the translocation channel is thought to be similar to that of *Escherichia coli* [121]. The mechanism of SecA-dependent translocation of secretory protein through SecYEG channels was reported by Kudva *et al*. [56]. The overexpression of a constructed artificial *secYEG* operon resulting in an increased number of translocons enhances the secretion amounts of the active exoprotein α-amylase [75]. The plasmid pAX01 containing *secY*, *secE*, and *secG*, which are under control of the xylose-inducible P_{xylA} promoter, integrates into the bacterial chromosome *via* a double crossing-over event at the *lacA* locus. SecDF is proposed to pull the pre-protein out of the SecYEG channel. Evidence resulted from the comparison of the structure but still lacks biochemical testing [68].

The homotetrameric SecB is employed as a targeting factor that binds to the core regions of the post-translated pre-proteins. It targets them to the SecA subunit of the translocase in order to maintain them in an unfolded translocation-competent state in gram-negative bacteria. Since *Bacillus subtilis* lacks the homolog to SecB, the 12 kDa homodimeric CsaA fulfills a similar function to SecB in *Escherichia coli* [96] for a certain subset of pre-proteins, including PhoB [76] and YvaY [62]. Defect of SecA in *Escherichia coli*, affecting normal growth and protein secretion, can be suppressed by chaperone-like activities of *Bacillus subtilis'* CsaA. This is consistent with the fact that CsaA is significantly induced to combat secretion stress

when α-amylase is highly expressed [41]. It remains to be an ongoing subject of study to elucidate the exact mechanism of CsaA, even after determination of crystallographic details.

SecA, a dual functional element, acts as a co-chaperone as well as the motor component of the Sec translocase. Even though the structure of SecA has been reviewed by Papanikou *et al*. [83] and Rocak and Linder [92] in detail, the mechanism of its function is still unclear. Here, we summarize some research on it. Zimmer *et al*. [131] reported on different dimers of SecA, indicating that they are all in equilibrium with monomers and do not differ dramatically in their stability. Moreover, and regarding the X-ray structure of *Bacillus subtilis* SecA, they assume that this monomer is required for protein translocation. One commonly known protein-conducting channel consisting of SecA, SecYEG, and SecDF has high affinity. The other low-affinity channel is composed by SecA alone. Pre-proteins can be transformed into the high-affinity channels owing to the lower specifity and less efficiency of SecA oligomers [111]. Both the tail-to-head tandem dimer SecAA and the tail-to-head SecAAA trimer complement a temperature-sensitive SecA mutant, even though these purified dimers and trimers show low-level intrinsic ATPase, similar to soluble monomeric SecA. Nevertheless, their activity can be improved to the same extent as the wild-type SecA protomer when stimulated by liposomes. It also was suggested that SecA may exist and function as a hexamer in the membrane.

In order to make full use of SecA and to improve secretion, many efforts have been made, either changing SecA itself or other components interacting with SecA. Kakeshita *et al*. [44] examined the influence of modifications in the C-terminal region of SecA on recombinant protein production in *Bacillus subtilis*. The C-terminal domain of SecA includes the poorly conserved C-terminal linker (CTL) and the highly conserved SecB-binding site, even though there is no SecB in *Bacillus subtilis*. The CTD of SecA seems not to be essential for heterologous protein secretion. When the 66 amino acids of SecA's C-terminal are deleted, there is no difference between the SecA mutant and the wild-type strain. Not only is the cell viability normal, but the activity of the thermostable heterologous alkaline cellulase (Egl-237) also increases by 83%. Human interferon-α (hIFN-α2b) productivity increases about 120%. Furthermore, Diao *et al.* [20] constructed a co-expression system of SecB from *Escherichia coli* and a hybrid SecA in *Bacillus subtilis*. At the same time, the last 32 amino acids at the C-terminal of SecA were replaced by the corresponding part from *Escherichia coli's* SecA, thus admitting the artificial SecA combined to SecB. A dramatic improvement of exported protein production took place when assessing the efficiency of the system. All these studies pave the way for future understanding of the mysterious translocase.

Removal and degradation. Third, the cleavage of N-terminal signal peptide sequences is accomplished by SPPases. Then, SppA and TepA are recruited for the subsequent degradation of the cleaved signal peptides. Otherwise, the cleaved signal peptides may become inhibitors to other protein translocation.

The SPPases in gram-positive species of *Bacillus subtilis* are divided into two types with respect to their distinct substrate specificity: type I (Lep) for most secretion proteins and type II (LspA) for lipoproteins. Five of seven *sip* genes for type I signal peptidase (denoted as *sipS*, *sipT*, *sipU*, *sipV*, and *sipW*) have been identified to locate on the chromosome. The presence of either SipS or SipT is sufficient for processing of precursor proteins and cell viability [104]. Multiple SPases may serve to guarantee a flexible capacity for protein secretion under various conditions. For example, SipW is a bifunctional signal peptidase, and is not only requested for the processing of TasA, which is a spore-associated protein [105] but also controls surface adherent biofilm formation [100]. The other two genes encoding SipP are part of homologous structural modules presented on at least two cryptic plasmids, pTA1015 and pTA1040. Their transcription product SipP can functionally replace SipS and SipT [106]. Since type II is responsible for lipoprotein cleavage, however, processing of lipoproteins by LspA is not strictly required for lipoprotein function [52]. The subject of SPase I and SPase II deletions has been reviewed by Tjalsm *et al.* [102] in order to further elucidate the effects of the signal peptidases.

The signal peptide peptidase SppA, a membrane-bound protein, is considered to be merely required under special conditions, such as hypersecretion [9]. Recent studies showed that SppA self-processes its own C-terminus, and proposed a model for how the C-terminus of SppA may function in the regulation of this membrane-bound self-compartmentalized protease [78]. Unlike SppA, TepA seems to be located in the cytoplasmic matrix. TepA depletion has a significant effect on pre-protein secretion across the membrane and subsequent processing not only under conditions of hypersecretion [9].

Modification and maturing. Fourth, the translocational protein modification, folding process, and following procedures are catalyzed by factors including chaperones, pro-peptides, the peptidyl-prolyl *cis*/*trans* isomerase PrsA,

disulfide isomerases BdbBCD, metal ions, and a quality control system.

Bacillus subtilis has intracellular and extracytoplasmic chaperones mediating protein folding, decreasing aggregation, and maintaining pre-proteins in translocation-competent conformations. The intracellular chaperone series of GroE and DnaK are regulated by the *groE* operon (*groES–groEL*) and the *dnaK* operon (*hrcA-grpE-dnaK-dnaJ-yqeT-yqeU-yqeV*) [36, 59]. Except for these functions and summarized in publications [90] and patents [79] (mentioned above), it was reported that the chaperones DnaK and GroEL are recruited to membranes for early membrane protection under short-term ethanol stress conditions [95]. In *Bacillus subtilis*, CsaA seems to act as a chaperone by preventing their aggregation, in contrast to SecB in *Escherichia coli*.

The membrane-anchored lipoprotein PrsA belongs to the extracellular chaperones and is indispensable for normal growth by facilitating the mature protein to fold into its stable and correct conformation. Overexpression of the substrate-specific chaperone PrsA results in a dramatic increase of α-amylase production [14, 109]. Consistent observations occur when AmyE pro-peptide is fused to human interferon-β. In this case, and under co-expression of PrsA, the recombinant protein production increases by more than 1.5-fold [45]. In addition to this, it is suggested that the folding of penicillin-binding protein is dependent on PrsA, as the deletion of PrsA leads to unstable PBP2a, PBP2b, PBP3, and PBP4 [40]. Reporter assays showed that extracytoplasmic chaperones act after intracellular chaperones; that is, in a sequential manner when assessing the MH-1 (fibrin) SCA production [117].

The formation of disulfide bonds, catalyzed by thiol-disulfide oxidoreductases BdbBCD, is necessary for the stability and activity of many exo-proteins [10]. Overexpression of these thiol-disulfide oxidoreductases could improve secretion of proteins containing certain disulfide bonds, as reviewed by Nijland and Kuipers [79]. However, this increase was not significant, thus leading to the occurrence of a novel approach. To achieve an increased production, one should combine depletion of the major cytoplasmic reductase TrxA, introduction of the heterologous oxidase DsbA from *Staphylococcus carnosus*, and addition of redox-active compounds to the growth medium [53].

Partly misfolded proteins may aggregate into inclusion bodies both in and out of the cytoplasm. The amount of these cytoplasmic insoluble aggregates can be decreased by ClpCP, ClpXP, and ClpEP [37]. In *Bacillus subtilis*, the genes coding for ClpC, ClpE, and ClpP are members of the CtsR regulon (class of three stress gene repressors) except for ClpX [19]. ClpP is essential for intracellular protein quality control [50]. ClpE is involved in global protein disaggregation [71].

The quality control proteases consist of the two membrane-anchored proteins HtrAB and the cell wall-bound protease WprA [17]. All of them are considered to be responsible for inducing degradation of incorrectly folded proteins that had passed the membrane. The two-component system CssRS (control secretion stress regulator and sensor) controls the expression of the *htrA* and *htrB* genes in response to the stress induction due to the overexpression of both homologous and heterologous secretory proteins [39]. However, recent observations suggest that HtrAB, being indeed the substrate of multiple extracellular proteases [55], is crucial for maintaining the integrity of the *Bacillus subtilis* cell even under non-stress conditions [54].

Tat Pathway

Different from the Sec pathway, the Tat pathway transports fully folded proteins containing a conserved region of twin-arginine in the signal peptide sequences. The Tat pathway facilitates proteins that have been folded too fast and tightly in the cytoplasm to be compatible to the Sec pathway. At the same time, both cofactors and complex redox enzymes, which are involved in the respiratory chain and insert into the cytosol prior to transport, are necessary.

The basic procedures include these steps: First and before translocation, pre-proteins fold in the cytoplasm with the help of cofactors. Second, owing to the presence of a pH gradient across the cytosolic membrane, pre-proteins are transported through the Tat translocase [91]. Third, after translocation, type I Spases cleave signal peptides. The remaining proteins secrete into the medium. To elucidate the mechanisms of this pathway, more attention needs to be paid to the details concerning processing, proofreading, and quality control of Tat substrates.

Unlike the large amounts of proteins secreted *via* Sec translocons, only three substrates of the Tat pathway in *Bacillus subtilis* have been identified: PhoD, YwbN, and QcrA. *Bacillus subtilis* contains two *tatC* genes, denoted *tatCd* and *tatCy*. Both *tatCd* and *tatCy* are preceded by *tatA* genes, denoted *tatAd* and *tatAy*, respectively. TatAdCd and TatAyCy act as the Tat machinery with the function of secreting the twin-arginine pre-proteins PhoD and YwbN, respectively [42]. The membrane protein QcrA is a novel Tat pathway-dependent substrate, unveiled by quantitative proteomic analysis [29].

The 3D model of TatAd of *Bacillus subtilis* observed from electron microscopy by Beck *et al.* [7] suggests that TatAd is

not able to form a transporting channel by itself because of its small diameter of the TatAd pore. The solution NMR structure of TatAd showed a transmembrane (TMS) and an amphipathic helix (APH) forming an L-shaped structure and a largely unstructured C-terminal tail containing a densely charged region (DCR) [38, 110]. Walther *et al.* [110] proposed a charge zipper mechanism based on the complementarity between the DCR and APH; thus, the protein being "zippered up" by seven salt bridges forming a hairpin that crosses the lipid bilayer. During the transport process, TatA is an early interacting partner of functionally targeted Tat substrates [26]. TatC plays a role as insertase for twin-arginine signal peptides [25]. In contrast to *Bacillus subtilis*, gram-negative bacteria possess Tat translocases comprising the three membrane proteins TatA, TatB, and TatC. Previous and recent studies have showed that beyond recognizing twin-arginine signal peptides, TatC mediates the transmembrane insertion of a signal peptide. This is followed by the translocation of the cleavage sites across the bilayer with a concerted regulation of TatB [25].

The Tat pathway has become an increasingly popular transporting pathway in virtue of exporting fully folded proteins of interest into the cultural medium, decreasing the costs of purification. Furthermore, much attention has been paid to the investigations of biotechnology application. For instance, Yang *et al.* [122] expressed the methyl parathion hydrolase (MPH), an organophosphate (OP)-hydrolyzing enzyme, adding the twin-arginine signal peptide of trimethylamine N-oxide reductase (TorA) from *Escherichia coli* to MPH in *Bacillus subtilis* [51, 65]. The subsequent investigation exhibited an effective degradation of OPs on vegetables by MPHs, which are exported by the Tat pathway, exclusively. In addition, neither cell growth nor cell viability is inhibited by the secretion of MPH. It was reported by Xia *et al.* [119] that the cytoplasmic thermostable β-galactosidase from *Geobacillus stearothermophilus* carrying a twin-arginine signal peptide of *Bacillus subtilis* phosphodiesterase shows a relatively high exporting efficiency in *Bacillus subtilis* through the Tat pathway. This efficiency decreases when lacking the signal peptide sequence at the N-terminus. Apart from the useful expression system of *Bacillus subtilis* itself, the TatAdCd system plays a key role for substitutes of the TatABC translocases in *Escherichia coli*. After the signal peptide of TorA is placed ahead of green fluorescent protein (GFP), the amount of extracellular GFP increases owing to the leaking outer membrane. Nevertheless, cell viability is not affected [3]. These findings provide potent transport approach methods for recombinant heterologous proteins in large-scale production.

ABC Pathway

ATP-binding cassette (ABC) transporters comprise a comprehensive family of proteins existing in all living organisms, ranging from bacteria to humans. Despite that all ABC transporters can either import or export a wide variety of structurally and functionally unrelated substrates, they all share one characterization: They all include two transmembrane domains (TMDs), defining the substrate-binding site, and two soluble nucleotide-binding domains (NBDs) acting as the motor domain. Unlike the diverse translocator units of the TMD, several conserved sequence motifs have been identified in the NBD [35]. However, the molecular mechanisms of ABC transporter proteins remain unclear.

These ABC transporters implement their functions in different aspects. Here, we list a part of recent advances in this field: *Bacillus subtilis* holds a set of five osmotically inducible osmoprotectant uptake systems, the Opu family of ABC transporters. Among them, OpuA is the main glycine betaine uptake system. The intracellular solute pool plays a key role in the osmotic control of OpuA expression [34]. The crystal structure of the OpuBC/choline complex, which is the substrate-binding protein of the OpuB transporters, provides a rational explanation for the observed choline specificity of the OpuB ABC importer *in vivo* [89]. Different from OpuB, structures of the substrate-binding proteins give insight into the multi-compatible solute-binding specificities of OpuC [22].

There are also many response regulators and operonic kinase-regulator pairs in *Bacillus subtilis*. Close relationships exist between some operons encoding two-component systems and genes for ABC transporter homologs. The *natAB* operon, encoding a two-gene ABC transporter and involved in sodium ion extrusion, has been identified to be regulated by the NatKR two-component system [80]. The FtsEX ABC transporter regulates cellular differentiation in initiating sporulation [28]. YheI/YheH form a new heterodimeric multidrug ABC transporter and are predicted to be possibly responsible in multiple antibiotic resistance [107].

Transformation Methods and Genetic Engineering Strategies

Transformation Methods

All of the genetic engineering strategies are based on the development of high-efficiency transformation methods. The usual approaches comprise the competent transformation method [98], the protoplast transformation method [13],

the protoplast electroporation method [57], the alkali metal ions method [4], and different kinds of electroporation methods and strategies based on the restriction modification system.

According to the competent transformation method, *Bacillus subtilis* enters a physiological state after incubation in GMI and GMII media, permitting the uptake of exogenous DNA. This process is controlled by a complex signal transduction cascade. This classical method is easy to manipulate, does not require special machines, and also results in high transformation frequencies.

The protoplast transformation method, nevertheless, is time consuming and labor intensive, owing to the protoplast preparation by degrading the cell wall and requiring experience on manipulation. In addition, cell regeneration takes several days. However, it is a useful transformation method, especially when the bacteria are difficult to be transformed owing to the physiological barrier.

Electroporation is a simple and widely used technique for transformation of various *Bacillus* species. Adding sorbitol, mannitol, or glycerol in hyperosmotic concentrations in the electroporation medium as well as in the growth and recovery media enables extremely high electric field strengths and improves transformation efficiencies [120]. Protection by high osmolarity media during electro-bombardment improves cell survivability. In addition, trehalose [67], sorbitol and mannitol decrease damage of electric shocks to the cells when they are added to electroporation media. In *Bacillus subtilis*, this results in increased transformation efficiency by nearly 100-fold compared with conventional methods. Apart from adding these osmoticums in order to balance the high internal turgor pressure of bacterial cells, adding glycine and D/L-threonine weakens cell walls even more. It is another strategy to elevate the electrotransformation efficiency, as well as cell-membrane fluidity when adding Tween 80 [128].

Moreover, the *in-vivo*-methylation strategy improves transformation efficiencies by methylation of exogenous plasmid DNA before transforming undomesticated strains or strains containing strict restriction and modification systems (RMS) [27]. Not only can the plasmids be methylated to deny the restriction enzymes, but also the restriction system in *Bacillus* can be modified. Deletion of two type I RMS of the *hsdR* loci creates a library of readily transformable [113] mutants of *Bacillus licheniformis* DSM13. This may also be applicable to *Bacillus subtilis*. Overexpression of the competence master regulator ComK is another alternative to make host strains become supercompetent cells. The transformation efficiency is highly improved when multimeric plasmids, generated by using prolonged overlap extension-PCR, are transformed [130]. There is also an approach based on conjugational plasmids, which is able to burden extra DNA and transmit it to another *Bacillus* [81].

Genetic Engineering Strategies

There has been much progress in the development of genetic modification strategies of *Bacillus* species (Dong and Zhang [21]). The operator-repressor system based on genetic engineering strategies includes CI repressors from *Escherichia coli*, bacteriophage λ and *araR*. *araR* is a negative regulator of the *ara* operon that can be induced by L-arabinose in *Bacillus subtilis* as a counter-selection marker. *upp* and *pyrF*, encoding uracil phosphoribosyltransferase (UPRTase) and orotidine 5′-phosphate decarboxylase (OMPdecase), have been used as counter-selection markers. These genetic engineering strategies make use of the pyrimidine metabolism. In addition, there are genetic engineering strategies in different *Bacillus* species based on auxotrophy, site-specific recombination, toxic genes, thermosensitive plasmids, and transconjugation.

In conclusion, taken together, increasing information about *Bacillus subtilis* in both basic research and application demonstrates that this potential industrial bacterium will become a super-secreting cell factory through continuous research and understanding [47], particularly in terms of the Sec and Tat pathways. Further studies are still needed in order to understand the function of each element of the Sec secretory machinery and their interaction. Further focus on the Tat pathway seems indispensable, since it enables folded proteins to travel through the membrane, ensuring the bioactivity of products. Attracting further attention, the network between the different pathways allows robust bacteria to overcome unfavorable environmental conditions and pass the bottlenecks of secretion.

Apart from the advances in elucidation of complicated mechanisms and control of the Sec and Tat pathways, there may be other aspects to increase secretion efficiency (Fig. 3): the development of protease-deficiency strains, minimizing the unnecessary genome sequences [69, 70, 74], and optimization of metabolism engineering techniques. Harness of the systems and synthetic biological approaches can improve industrial production, with the combination of genomics and proteomics. In conclusion, it is crucial to investigate the mechanisms of the secretion pathways and the influencing factors in order to broaden the range of efficient industrial application of *Bacillus subtilis*.

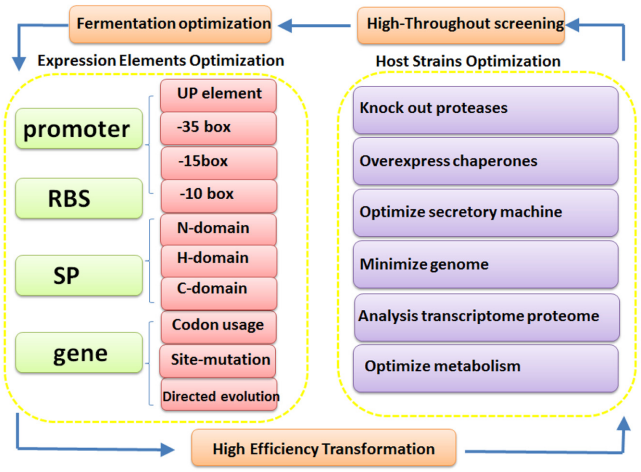

Fig. 3. Scheme of strategies to improve the protein secretion production by *Bacillus subtilis*.
RBS, ribosome-binding site; SP, signal peptide.

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