REVIEW

Molecular engineering of secretory machinery components for high-level secretion of proteins in *Bacillus* species

Zhen Kang · Sen Yang · Guocheng Du · Jian Chen

Abstract Secretory expression of valuable enzymes by *Bacillus subtilis* and its related species has attracted intensive work over the past three decades. Although many proteins have been expressed and secreted, the titers of some recombinant enzymes are still low to meet the needs of practical applications. Signal peptides that located at the N-terminal of nascent peptide chains play crucial roles in the secretion process. In this mini-review, we summarize recent progress in secretory expression of recombinant proteins in *Bacillus* species. In particular, we highlighted and discussed the advances in molecular engineering of secretory machinery components, construction of signal sequence libraries and identification of functional signal peptides with high-throughput screening strategy. The prospects of future research are also proposed.

Keywords Signal peptide · Bacillus subtilis · Secretory machinery · Enzyme production · Cell factory

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Introduction

Since the advent of recombinant DNA technology, numerous industrial enzymes such as proteases, amylases and lipases have been produced by Gram-positive bacteria and fungi. Due to many advantages including powerful capacity for protein secretion [6, 62, 64, 69, 76], non-pathogenicity [76, 84], high amenability for genetic engineering and large-scale fermentation [25], *Bacillus* species (especially *Bacillus subtilis*) have been intensively modified for the production of pharmaceutical proteins and enzymes (Table 1). To date, four distinct routes [25] (the Sec pathway, the twin-arginine translocation pathway, the ATP-binding cassette transporters and a pseudopilin export pathway) for protein export have been characterized and documented in *B. subtilis*. Among them, the largest and most distributed Sec pathway has been intensively and deeply studied [17, 64]. In general, the secretion process can be divided into three steps: targeting, translocation and release. Proteins (enzymes) secreted via this pathway are synthesized as precursors with N-terminal signal peptides (SPs). By interacting with many other molecular chaperones, SP directs the precursor to be recognized and digested by signal peptidase (SPase). Subsequently, the mature protein is released into extracellular medium with enzymatic function [56]. As a consequence, all the secretory machinery components including SP play crucial roles in the process of translocation and secretion.

Although several heterologous enzymes have been successfully produced by *B. subtilis*, the titers of some recombinant enzymes are still low to fulfill the requirements of practical applications [76]. As a result, it is imperative to explore the secretory process and engineer the secretory machinery components to improve the secretory efficiency. Herein, we describe recent achievements in the exploration

Table 1 Secretory expression of protein products from *Bacillus subtilis*

Product	Signal peptides	Titers	References
Bacillus subtilis			
Proinsulin	NprE	1 g/L	[59]
Interleukin-3	Lat	100 mg/L	[83]
MH-1 SCA	Levansucrase	10–15 mg/L	[88]
hEGF	Staphylococcal protein A	7.0 mg/L	[44]
α-amylase	SacB	2012 U/mL	[27]
Cutinase	LipA	20 mg/L	[7]
Methyl parathion hydrolase	NprB	27.1 U/mL	[99]
Mannanase	amyQ	8.65 U/mL	[23]
Lipoxygenase	NprB	76 U/mL	[96]
Alkaline polygalacturonate	Bpr	620 U/mL	[98]
Dextranase	ImdA	4.6 g/L	[26]
Lipase	SacB	356.8 U/mL	[47]

and molecular engineering of the secretion machinery components. We give particular attention to novel strategies such as SP library construction and high-throughput screening (HTS) that applied for increasing the secretion capacity. Future perspectives in achieving coordinated balance between protein expression and secretion are also proposed.

Secretory machinery components and its engineering

Structures of SP

SP, a regulatory element that locates at the N-terminus of secretory protein is essential for correct targeting to the translocation machinery and transportation from cytoplasm into growth medium. Although different SPs show no conserved amino acid sequence, three distinct regions can be summarized (Fig. 1a). Especially in the Sec-type SP, the positively charged amino-terminal region (N-region) always contains two or three lysine (L) or arginine (R) residues which are responsible for determining the final orientation of SPs in membrane [64]. The central hydrophobic core (H-region), which generally preserves a helix-breaking residue glycine or proline at position −4 to −6 relative to the cleavage site, is believed to adopt an α-helical conformation in membrane and is critical for early stages in protein export [95]. In parallel, the polar carboxyl terminal region (C-region) which commonly ends with the type I SPase recognition sequence Alanine-X-Alanine (AXA) is considered to adopt a β-stranded conformation [60, 72]. In view of these characteristics, programs such as SignalP

[55] and LipoP [32] for the prediction of SPs have been developed and widely applied.

To date, SPs have been considered to be endowed at least three functions. First, SPs should inhibit the folding of intracellular nascent chains to retain translocation competence and avoid the activation of potentially harmful secretory enzymes [11, 77]; Second, SPs should interact with other secretion machinery components and direct the transmembrane process [12]; Third, SPs should serve as a topological determinant for proenzymes in the membrane (SPs initiate translocation of the polar C-terminal regions of proenzymes while the N-terminal regions of the SP remains behind the membrane) [18, 77].

Recently, many studies have confirmed that non-optimal codons enriched at the N-terminus are functionally important for protein conformation and activity [9, 22, 65, 93]. In prokaryotic cells, it has been believed that folding of proteins is synchronous with their biosynthesis at the ribosomes, and non-optimal codons are often installed at the domain boundaries to slow the translation rate [40]. This local discontinuous translation enables the nascent chains having plenty of time to correctly fold into specific domains [86, 97]. In correspondence, many non-optimal codons have also been found in the SP regions of a number of secretory proteins [65, 91]. Interestingly, Zalucki et al. [92, 93] reported that artificial optimization of the natural non-optimal codons significantly decreased the secretion of maltose-binding protein (MBP) and β-lactamase (Bla). The results suggest that usage of non-optimal codons slows the rate of translation and facilitates proper folding of the secreted protein. More recently, Goodman et al. [21] further demonstrated that N-terminal rare codons are beneficial to reducing RNA structures, which finally lead to increased expression of the corresponding genes. As a consequence, artificial engineering of SP by introducing rare codons might be an attractive approach to improve the secretory production of target proteins.

Signal recognition particle

Signal recognition particle (SRP) (Fig. 1b), the ancient and highly conserved ribonucleoprotein complex (Fig. 1b), is in charge of recognizing the SP sequence of a nascent chain and targeting it to the membrane. In *B. subtilis*, the SRP complex is composed of a small cytoplasmic RNA (scRNA) [52], two GTPases (Ffh and FlhF) [53, 72, 94] and a histone-like protein (HBsu) [53]. In addition to the above components, FtsY, the SRP receptor that belongs to the widely conserved SRP–GTPase family, is also essential for transferring the ribosome nascent chain complex to the translocation channel [58]. Previously, it has been discovered that CsaA (Fig. 1b), which seems to serve as a SecB homologue [5], can directly interact with SecA and
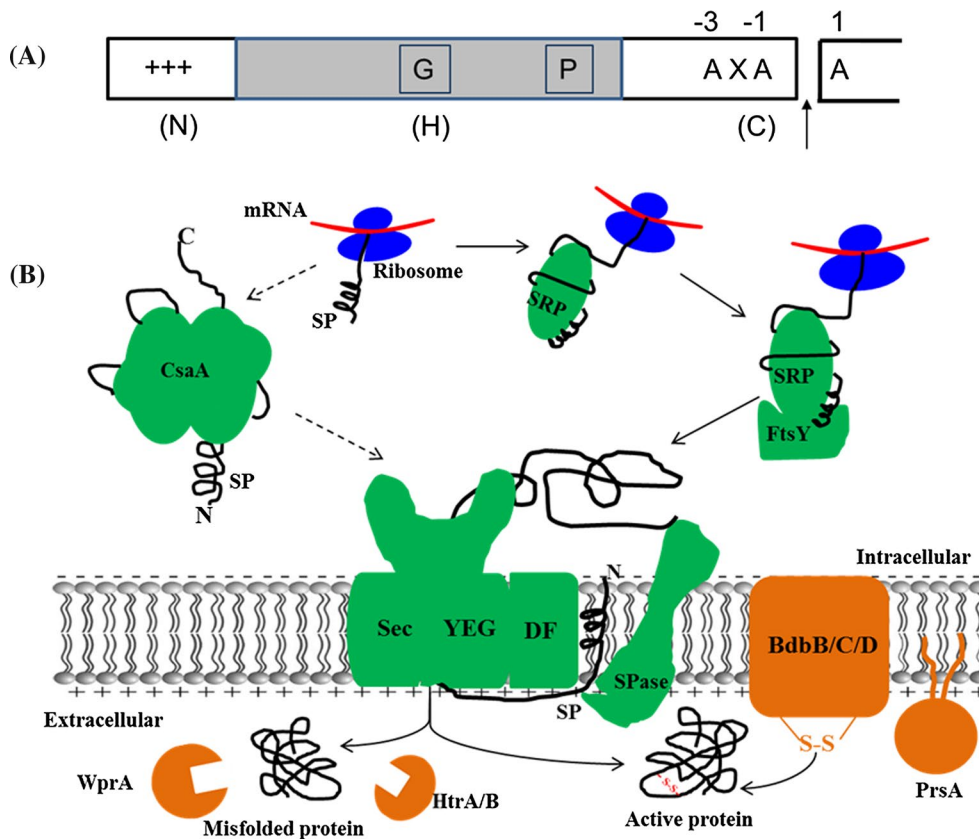
Fig. 1 Schematic representation of putative protein secretory machinery in *B. subtilis*. **a** Tripartite structure of the Sec-type signal peptide (SP): a positively charged N-terminal (N), a hydrophobic domain (H) and a cleavage domain (C) with specific cleavage site (*arrow*). The boxed P and G mean the helix-breaking residues proline and glycine. The numbers represent the positions relative to the cleavage site; **b** The Sec pathway and key regulatory components in *B. subtilis*. Two putative translocation pathways are represented (CsaA-SecA pathway in *dashed black arrows* while SRP-SecA pathway in *solid black arrows*). The mRNA was represented in *red*. The ribosome complex was in *blue* while the nascent peptide was in *black*. The signal recognition particles (SRP), CsaA, FtsY, SecA and signal peptidase which interacted the SP are shown in *green*. The regulatory proteins that involved in folding of secretory proteins (PrsA, BdbB/C/D) and the degradation of misfolded protein are highlighted in *brown*. The disulfide bonds in active proteins are shown in *red*

precursor proteins to influence the secretory efficiency [51]. In view of this result, Diao et al. [16] recently constructed an artificial protein targeting pathway by co-expressing SecB (*E. coli*) and a *B. subtilis* hybrid SecA (the C-terminal 32 amino acids were replaced by the corresponding fragment of SecA from *E. coli*), and successfully increased the secretion of both maltose-binding protein (MalE11) and alkaline phosphatase (PhoA) (Table 2). The results suggest that modification of the SRP components is a potential novel strategy for improving the secretory capacity of *B. subtilis* and its related species.

Translocase complex

In bacteria, the Sec-translocases (Fig. 1b) are conserved and consist of a heterotrimeric protein SecYEG complex (core) [14], SecA (motor) [29, 72], a SecDF–YrbF complex [3] and YidC homologues (SpoIIIJ and YqjG)

[89]. The structure and interaction mechanism of bacterial Sec-translocase and translocation process have been well reviewed [14, 17, 56, 90]. In general, the limited secretory efficiency is attributed to the insufficient capacity of the transport machinery, especially the translocation process [10]. To improve the translocation capacity, many mutation studies have been performed on the translocator complex to identify the key elements involved in protein translocation and secretion [28]. SecA, the motor protein, is considered to be a key regulator since its ability to directly interact with both the precursors and membrane translocases. On this base, Kakeshtia et al. [33] successfully enhanced the extracellular production of heterologous proteins in *B. subtilis* by deleting the C-terminal region of SecA. The results notably suggest that modification of the Sec-translocase components is an efficient approach for improving the secretory capacity of *B. subtilis*. In many cases, overexpression of secreted

Table 2 Improvement of protein secretion via engineering translocation machinery components

Components	Natural Function	Modifications	Target proteins	References
SRP				
CsaA (SecB)	Chaperone for recognition and transportation the new nascent polypeptide	Coexpression of SecB and a hybrid SecA	MalE11and PhoA	[16]
Translocase complex				
SecA	Translocation ATPase	Deletion of the C-terminal region	hIFN-α2b	[33]
seYEG	Integral membrane proteins for translocation	Expression	α-amylase	[50]
Signal peptidase				
SipM	Signal peptidase	Expression	DsrS	[48]
Regulatory factor				
PrsA	Regulatory protein for folding of the new nascent polypeptide	Expression	Subtilisin	[37]
		Expression	rPA	[85]
		Expression	α-amylase	[79]
BdbD (DsbA)	Thiol-disulfide oxidoreductases	Modulation of	PhoA	[38, 39]
WprA	Serine protease for extracytoplasmic quality control	Deletion	rPA	[63]
GroEL–GroES	Chaperones involved in minimizing aggregation of the new nascent polypeptide	Expression	SCNF	[87, 88]

SRP Signal recognition particle, *MalE11* maltose-binding protein, *PhoA* alkaline phosphatase, *hIFN-α2b* human interferon α, *DsrS* Dextransucrase, *rPA* recombinant protective antigen, *SCNF* Single-chain antibody fragment

proteins can cause jamming of membrane because of the shortage of translocons. To solve this bottleneck and increase the number of translocons, Mulder et al. [50] firstly constructed an artificial *secYEG* operon to optimize their expression and substantially increased the secretory production of α-amylase (Table 2). The results evidently confirmed that balanced expression of translocase components is crucial for efficient translocation and secretion.

Signal peptidases

Bacillus subtilis owns two types of SPases for the cleavage of substrate proteins [25]. The type I SPases are responsible for cleaving most classes of secretory proteins while the type II SPases are exclusive for lipoproteins [74]. Generally, five conserved regions are preserved in type I SPases from bacteria to human [13, 60, 61]. Unlike *E. coli*, *B. subtilis* has five chromosomal SPases (SipS, SipT, SipU, SipV and SipW) [72]. SipS and SipT are the key SPases for protein secretion while others should be required for its flexibility and viability [73]. Once the secretory protein translocated, the C-region of the transmembrane SP would be recognized and quickly deleted by SPase [77]. Recently, high-resolution crystal structure of the SPase A from *B. subtilis* not only provides new insights into its function mechanism but also promotes its rational engineering [54]. In fact, Malten et al. [48] have successfully increased the secretory production of recombinant proteins by overexpressing the type I SPase in *B. megaterium* (Table 2).

Regulatory factors

To accomplish efficient secretion and correct folding, many molecular chaperones, foldases and quality control proteases are preserved in *B. subtilis*. Research progress regarding the intracellular chaperones GroES, GroEL, DnaK, DnaJ, GrpE and CsaA and the only extracytoplasmic folding factor PrsA have previously been well reviewed [18, 46, 68]. In all Gram-positive species, the membrane-associated lipoprotein PrsA (Fig. 1b) is ubiquitously distributed with about 20,000 molecules per cell [37, 80]. Related experiments in *B. subtilis* have shown that inactivation of PrsA resulted in increased rates of degradation and loss of enzymatic activity [31, 85] while overproduction of PrsA significantly enhanced the secretion of active subtilisin [37], rPA (recombinant protective antigen) [85] and α-amylase [79] (Table 2). In this respect, PrsA should be a potential engineering target for the secretory production of target proteins.

In *B. subtilis*, four thiol-disulfide oxidoreductases BdbA, BdbB, BdbC and BdbD have been identified [4, 49] and well reviewed [68]. To increase the formation of disulfide bonds in heterologous proteins, up-regulation or modification of these thiol-disulfide oxidoreductases should be essential since very few disulfide bonds exist in natural secretory proteins of *B. subtilis*. To date, many proteins containing disulfide bonds have been successfully produced in *B. subtilis* by modulation of thiol-disulfide oxidoreductases [38, 39]. In addition, many quality control proteases like HtrA, HtrB and CWBP52 (WprA) (Fig. 1b)

that regulated by the two-component system CssR–CssS have been reported and designated for degradation of misfolded or aberrant proteins in *B. subtilis* [30, 57, 70]. The structure and function mechanism of these proteases have been well studied [34–36, 42]. In particular, it was discovered that the HtrA family proteins HtrA, HtrB and WprA are involved in protein quality control and stress response [24, 41]. For this purpose, Pohl et al. [63] investigated the *B. subtilis* mutants with deletion of HtrA, HtrB or WprA at proteomic level and found that inactivation of WprA improved the production of heterologous proteins. These results suggest that regulatory factors involved in protein secretion, quality control and stress response are the potential engineering targets for sensing and indicating the concentration of enzymes secreted by *B. subtilis*.

Novel approaches for identification of SPs and construction of its libraries

Prediction and identification of SPs with genomic and proteomic approaches

After completing genome sequence of *B. subtilis* 168 [43], all putative SPs were predicted from the annotated *B. subtilis* proteins in the SubtiList database (http://bioweb.pasteur.fr/GenoList/SubtiList) with SignalP algorithm [55, 72]. Subsequently, functional genomic and proteomic approaches were also employed to explore the secretome of *B. subtilis* [1, 71].

In addition to *B. subtilis*, *B. licheniformis* as the other important *Bacillus* species has also been extensively investigated after its complete genome sequence [67, 78]. In 2006, Voigt et al. investigated the extracellular proteome of *B. licheniformis* under different nutrient starvation conditions [81]. Further comparative analysis results showed that *B. subtilis* possesses less Sec-type SPs compared with *B. licheniformis* [82]. In addition to *B. subtilis* and *B. licheniformis*, the extracellular proteomes of other *Bacillus* species including *B. cereus* and *B. anthracis* were also explored, and many extracellular proteins including putative virulence factors were identified [2, 20, 45]. More recently, Gohar et al. [19] comparatively studied the extracellular proteomes of *B. cereus*, *B. anthracis* and *B. thuringiensis*, and identified many novel SPs. Meanwhile, the results also indicate that the profiles of secreted proteins from *B. cereus* and *B. thuringiensis* are quite different from that of *B. anthracis*. With the development of genomic and proteomic approaches, more potential SPs will be identified and applied for secretion of the target proteins.

Construction of SP libraries for optimizing secretory expression

To achieve high-level production of the target enzyme, it is of utmost importance to choose an appropriate SP regulatory element. To this end, Brockmeier et al. [6] developed a novel and powerful strategy to optimize heterologous protein secretion in *B. subtilis*. Applying this systematic screening approach, all naturally occurring Sec-type SPs from *B. subtilis* were cloned to generate a library (Fig. 2b, natural) for HTS of the optimal SP toward the specific protein (Fig. 2d). Similarly, Degering et al. [15] recently constructed a bigger library (173 SPs from *B. subtilis* and 220
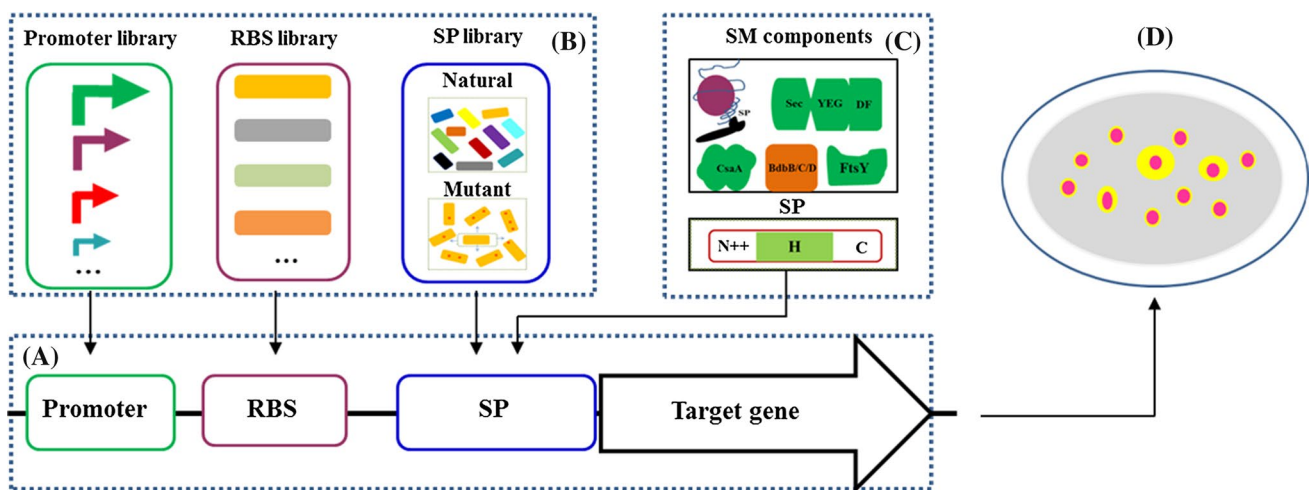

Fig. 2 Strategies for improving the secretory production of the target enzyme. **a** Regulatory elements for the expression and secretion of the target gene. RBS, ribosome binding site; **b** Constructed libraries of promoter, RBS and signal peptide (SP, natural and mutant); **c** Modification of the secretion machinery (SM) components. All the SM components could be rationally and simultaneously engineered in *Bacillus subtilis*; **d** High-throughput screening for the optimal SP toward the enzyme of interest

SPs from *B. licheniformis*) for HTS application. Although significant differences occur in Sec machineries and SPases between *B. subtilis* and *B. licheniformis*, majority of the homologous proteins from these two closely related strains showed comparable secretion levels. As a result, to achieve high-level secretion of the target enzyme in other *Bacillus* species, a genetically accessible host strain like *B. subtilis* can be used as a preliminary screening platform [15]. Recently, Caspers et al. [8] constructed a mutant library by saturation mutagenesis of the N-domain of the AmyE SP (Fig. 2b, mutant) and successfully screened several mutant SPs for improving the secretion of a heterologous model protein cutinase.

In many cases, application of the HTS approach might be constrained since not all enzymes can be easily characterized in vitro. Therefore, development of an in vivo universal method to directly evaluate the secretion capacity of each SP will be attractive. Accordingly, by analyzing the CssRS-mediated secretion stress response, Trip et al. [75] developed a novel HTS approach with green fluorescent protein as a reporter and realized rapid separation of the secreting cells from the non-secreting cells. In addition, this work also demonstrated that optimal balance between protein biosynthesis and the whole secretion process is crucial to the yield and quality of secreted proteins in *B. subtilis*. More recently, our results [98] also confirmed that excessive translation rate is adverse to the final production of secreted alkaline polygalacturonate lyase. As a result, achieving a coordinated balance between biosynthesis and secretion with combinatorial strategies (Fig. 2a, b) will be fascinating.

Concluding remarks

Although many natural SPs have been successfully characterized and applied to produce heterogeneous enzymes in *B. subtilis*, construction of powerful secretory machinery will be the prerequisite for achieving large-scale production of target enzymes. To date, many novel engineering strategies and powerful tools have been accumulated with the development of synthetic biology toward *B. subtilis* [66, 76], which further enable us to coordinate the balance between protein expression (including transcription and translation) and secretion (Fig. 2a, b). Meanwhile, deep understanding of the secretion regulation mechanism will also promote rational engineering of the secretory machinery components including SPs (Fig. 2c) toward the enzyme of interest.

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