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Review

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Bacillus subtilis Spore Surface Display Technology: A Review of Its Development and Applications

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Bacillus subtilis spore surface display (BSSD) technology is considered to be one of the most promising approaches for expressing heterologous proteins with high activity and stability. Currently, this technology is used for various purposes, such as the production of enzymes, oral vaccines, drugs and multimeric proteins, and the control of environmental pollution. This paper presents an overview of the latest developments in BSSD technology and its application in protein engineering. Finally, the major limitations of this technology and future directions for its research are discussed.

Keywords: *Bacillus subtilis*, spore surface display technology, biocatalysis, gene engineering, anchor protein, linker peptide

Introduction

Since the first report in 1985 on surface display systems, which used a filamentous phage-coat protein to immobilize antibodies on a phage surface [1], a number of surface display systems have been reported, such as for phages [2], yeasts [3] and bacteria [4]. However, despite the promising features of microbial surface display technology, these systems have not been found suitable for harsh industrial conditions, especially for expressing enzymes sensitive to such environments. On the other hand, the capability of bacterial spores to sustain extreme environments and maintain sporulation and germination cycles make them promising candidates for surface display technology.

Among the few spore-forming bacterial species, *Bacillus subtilis* is considered attractive for use in surface display technology due to its probiotic features and its generally-recognized-as-safe (GRAS) certification [5]. This gram-positive bacterium is well known for forming spores (endospores) under extreme conditions and, thus, can survive for long periods in harsh environments. Moreover, with the increasing amounts of knowledge about its structure and physiology that are being gained, *B. subtillis* spores have attracted much attention in bio-catalysis applications in recent years.

The utilization of *B. subtilis* spores for this purpose has been extensively reported since its first successful application in 2001 by Isticato *et al.* [6]. The basic principle of spore surface display is to enhance the functionality and stability of target proteins by anchoring such exogenous functional proteins on the surface of spores using various techniques. In this technology, a recombinant expression vector is usually constructed by introducing a target gene and gene-encoding spore coat protein (called an anchor protein) to its own promoter, which is then transferred to the host strain. The exogenous protein or polypeptide is expressed on the host spore's surface by inducing spore formation in a harsh culturing environment. These spores

have high resistance to extreme environments, and the exogenous proteins attached to the spore surface also gain such stress resistance.

B. subtilis spore surface display (BSSD) technology has been used for various purposes since its first application in displaying tetanus toxin (TTFC) by fusing *tet*C genes with the genes encoding the spore anchor protein CotB [6]. Nowadays, this technique is largely used in biodegradation, bio-catalysis, pharmaceuticals, industrial enzymes and production of other functional proteins [7, 8]. In view of the facts mentioned above, BSSD technology has attracted much research interest across the world. The objective of this review is to discuss the key aspects of spore surface display systems, focusing on this technology's technological improvements, applications, recent progress and future prospects.

Sporulation and Structure of Spores in *B. subtilis*

Adverse environmental conditions stimulate bacterial sporulation, in which spores are formed. At the initial stages, the replicated chromatin of vegetative cells is relocated to the poles of the cell with the help of RecA protein. The membrane then divides cells asymmetrically into pre-spore and mother cells by the activation of σ F and σ E, respectively, which stimulates the expression of related genes to produce pre-spores from the mother cells. After formation of pre-spores, a structure is formed around the bacterial cell wall with a thin layer of peptidoglycan by activation of two other factors, σ G and σ K. Finally, mother cells are lysed and mature spores are released by the action of autolytic enzymes [9].

The structure of mature spores, from the interior to the exterior, comprises a core, cortex, spore coat and exosporium. The spore coat can make spores resistant to chemical agents and its external lysozyme is composed of more than 70 coat proteins, including a base layer, inner coat protein and outer coat protein. The cortex of the spores has four layers. The cortex and core are the key factors for dormant sporulation and low moisture retention. The cortex mainly consists of peptidoglycan (PG) with a structure similar to that of the PG found in growing cells, but with a special modification. The spore core contains a bacterial genome and two unique components, namely, 2,6-dicarboxylic acid (DPA) and α/β small molecule acid-soluble protein (SASP) [10]. DPA, a component not found in vegetative cells, can replace water removed from the chromosome during sporulation to stabilize the spore macromolecules and enhance their heat resistance. SASP can cooperate with

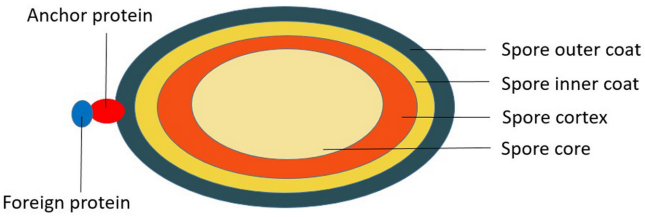

Fig. 1. The structure of a spore.

DPA to protect DNA from physical and chemical damage, especially from heat. As mentioned above, Moeller *et al.* [11] demonstrated that the stress resistance of spores is associated with their structural components.

B. subtilis Spore Surface Display Technique

The BSSD technique is one of the versatile tools in molecular biology, which can locate exogenous protein on the surfaces of spores through the fusion vectors using two genes encoding anchor protein and target protein. After transforming the recombinant vector into host strain *B. subtilis*, heterologous protein together with anchor proteins can be expressed on the surfaces of spores by inducing sporulation. The displayed heterologous proteins then gain the ability to be active in the extreme environment.

Compared to microbial cell surface display systems, spore surface display technology offers several advantages in anchoring heterologous proteins. First of all, it is easier for foreign proteins or polypeptides to be displayed on spore surfaces that are combined with the substrates in such a way that the enzyme is capable of acting on the substrate to a greater extent. The strong resistance of the spores improves the stability of the fused protein's activity in complex environments [53], as fused proteins are more stable than free ones. The spore formation process is simple and economical, in addition to the fact that it can keep target proteins active under extreme environmental conditions with easy regeneration to vegetative cells when needed. Moreover, a fusion protein displayed on the surface of a spore can be easily purified with a high recovery rate [12].

Heterologous proteins anchored on the spore surface do not require to be penetrated into the cell membrane, which, not only avoids incorrect folding and positioning problems but also ensures the structure and biological activity of foreign proteins. Besides, the presence of ATP-dependent chaperones can result in the correct folding of fusion proteins. Due to the large molecular weights of the

multimeric proteins that can affect cell membrane structures in other surface display systems, BSSD's characteristic of not requiring trans-membrane activity has advantages for correctly anchoring large molecular weight foreign proteins to the surfaces of spores. In addition, BSSD systems do not have any obvious codon bias.

Components of *B. subtilis* Spore Surface Display System

Generally speaking, a number of factors affect the efficiency of BSSD systems, including anchor proteins, target proteins, linkers, expression vectors and other experimental parameters. The BSSD technique has been progressed, to some extent, on the basis of the study of these factors.

Anchor Proteins

Anchor proteins are essential for successfully expressing foreign proteins on spore surfaces. Efficient anchor proteins require certain criteria, such as (1) a domain that ensures exogenous proteins can be displayed on the spore surface; (2) a strong anchor domain that can be fused with the exogenous protein to display the target protein firmly on the spore surface; (3) fusion proteins formed from anchoring and target proteins that do not affect any of their functions; (4) and the ability to resist hydrolysis by proteases in the medium or periplasmic space. Protease resistant protein can be determined by protease accessibility test [8].

In recent years, many anchor proteins have been reported for use in BSSD, including CotB, CotC, CotG, CotZ, CotX, CotY, CotA, OxdD, CotE, CotZ, CgeA and other capsid proteins. Of these, CotB, CotC and CotG have been studied in-depth. CotB was the first spore coat protein to be used in spore surface display technology, and different lengths of CotB have worked as anchor proteins to successfully locate exogenous proteins on the spore surface [6]. On the other hand, both CotC and CotG can employ any amino acid sequence as anchor motifs. CotB and CotC are mainly used to express antigens, while CotG is utilized to locate different enzymes on the spore surface when focusing on whole-cell biotransformation.

In selecting suitable anchor proteins, we should not only consider their location but also their abundance. The location and abundance of these three anchor proteins can ensure that the foreign proteins displayed on the spore surfaces have the greatest contact with the external environment; however, the excavation of novel anchoring motifs is necessary to provide more comprehensive functions. Accordingly, a new coat protein, CotZ, has been identified for use with this technology. It has been shown that CotZ can be used to express relatively large proteins, for instance, FliD (*Clostridium difficille* flagellin) [13] and DPEase (D-psicose 3-epimerase) [14].

With the exception of 43.4 kDa's lingerie shell protein OxdD [15], all other anchor proteins reported thus far are outer coat proteins. OxdD is only one that has been applied—in the preparation of animal feed probiotics—which indicates that there is much space for further research on using it to express high molecular weight oligomeric enzymes. OxdD can protect passenger proteins to a higher extent by minimizing the impact of the spore coat assembly process because of its relatively low abundance and internal localization. Based on the above, it can be readily concluded that inner-coat proteins have huge potential for expanding the range of capsid proteins used to improve spore surface display systems.

CotX is another spore coat structural protein of *B. subtilis*, which has been reported to have the ability to carry foreign proteins onto the spore surface by displaying green fluorescent protein (GFP). Researchers think that CotX-mediated β-Gal displayed on spores may be a promising strategy for lactulose production [16]. Even though CotX has rarely been mentioned in the literature compared to other anchor motifs, it has demonstrated its strength as a molecular tool for displaying exogenous proteins.

CotY is another coat protein that can be utilized as an anchor motif. One research report [17] has shown that CotY may be a good candidate for displaying functional proteins by expressing two model proteins on the spore surface of *B. subtilis*. CotE is another anchor protein that has been investigated in only two studies for displaying bacterial beta-galactosidase [18] and bacterial tyrosinase [19]. Likewise, CgeA has been investigated as an anchor protein in only a few recent studies [20]. CotA is one laccase that can be applied to directed evolution to increase substrate specificity [21].

Existing studies have usually focused on utilizing only one anchor motif to exhibit heterologous proteins or polypeptides on spore surfaces of *B. subtilis*. Recently, however, breakthroughs were made in two studies on BSSD, in which multiple proteins were used. For example, Negri *et al*. [13] successfully displayed a fragment of *Clostridium difficile* FliD protein by using CotB, CotC, CotG and CotZ as anchor motifs on the surface of *B. subtilis* spores. In another study, Iwanicki *et al*. [20] selected several coating proteins: CotB, CotC, CotG, CotZ and CgeA, which

can be constructed for recombinant spores. One of the current challenges in BSSD is how to optimize the application of existing anchoring proteins to better serve the display effect. So, another novel research path reveals that using multiple anchoring motifs as fusion partners is another potential direction for improving the efficiency of spore display. Iwanicki *et al*. [22] constructed budding display-integrated vectors based on CotC and CotG with the non-essential genes lacA and pyrD, respectively, and promoted the development of a multi-anchored protein display system.

Linker Peptides

Linker peptides can form stable helical structures to solve the problem of having a rigid structure between the anchor protein and target protein. Substantial research efforts have been made that show that inclusion of flexible linker peptides in constructing a recombinant vector is an effective way to regulate the function of fusion enzymes. The linker peptides reported so far are GGGEAAAKGGG, GGGGS, EAAAK and AAAAAAAAAA. The first two have been widely used in earlier studies, while the linker AAAAAAAAAA (10 alanine residues) has only been used by Potot *et al*. [15] to assist in the expression of phytase and β-glucuronidase.

Hinc *et al*. [23] successfully placed the linker peptide GGGEAAAKGGG between the C-terminus of the anchor protein and the N-terminus of the exogenous protein UreA. Compared with using GGGGS as the linker, it was speculated that an α-helix could be formed in the GGGEAAAKGGG peptide chain, while the EAAAK structure was found to be crucial for improving the display efficiency. Quantitative comparison of the efficiency of displaying fusion proteins in CotZ-UreA and CotB-GGGEAAAKGGG-UreA showed that the former can only express 2.5×10^2 recombinant molecules on one spore surface, but the latter can be as high as 1.0×10^4. Huang *et al*. [24] used flexible units of GGGGS (F) and rigid units of EAAAK (R) to combine a completely rigid RRRR and a fully flexible FFFFF for constructing an acid phosphatase-green fluorescent protein, PhoC-GFP. They found that the highest activities occurred neither with the rigid RRRRR linker nor with the flexible FFFFF linker, but with the FFFRR linker. Their results showed that utilizing a linker library with the controlled flexibility of fusion proteins is an efficient way to regulate the relative specific activity of fusion enzymes. The results of the above studies indicate that it is necessary to apply high flexibility linker peptides for improving the efficiency of *B. subtilis* spore display systems.

Strategies of *B. subtilis* Spore Surface Display Systems

Fusion between Target and Anchor Proteins

Fusion of exogenous and anchor proteins can be achieved by involving the N-terminal, C-terminal and sandwich structures of the proteins. The fusion method is determined through the direction of anchoring during the process of

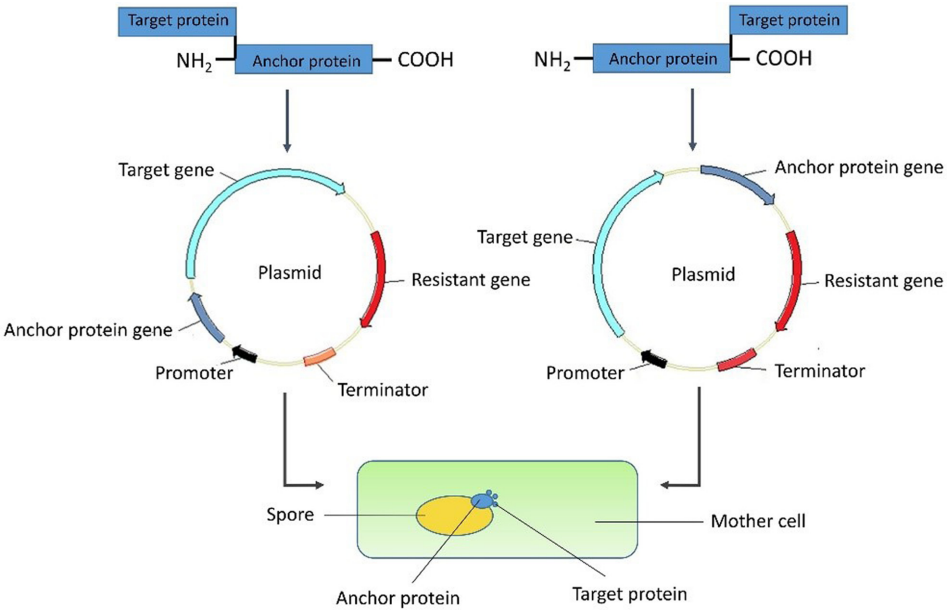

Fig. 2. Anchor and target proteins are fused via the N-terminal and C-terminal methods.

sporulation, which locates a target protein on the spore surface being expressed with anchor proteins. A set of 16 vectors were designed by Iwanicki *et al*. [20] for the spore surface display of heterologous proteins, and they concluded that CotB, CotC and CotG enable the receiving of N-terminal and C-terminal fusions, while the other two anchor proteins, CotZ and CgeA, can produce C-terminal fusions only. In another study, TTFCs were linked to the C-terminus, N-terminus and middle part of the anchoring protein CotB [6]. For other anchor proteins, such as CotG, CotZ, CotX and OxdD, display of foreign proteins is usually done via C-terminal fusion.

Recombination Strategies

BSSD can be conducted by recombinant and non-recombinant fusion approaches [8]. In the non-recombination spore surface display technique, bacterial spores are cultured with purified foreign proteins in a container to anchor them on the spore surface using electrostatic interactions, hydrophobic interactions, or cross-linkers such as glutaraldehyde [25], while vectors are constructed in the recombinant approach. Usually, two types of vectors are used in the construction of recombinant proteins, such as episomal vectors and integrating vectors.

The method of recombination is mostly based on the fusion of the genes encoding the foreign and anchor proteins, using either integrated or episomal plasmids. Along with the induction of the spore formation process, foreign proteins are successfully displayed on the spore surface without affecting the structure and function of the spores. Episomal vectors that replicate independently can be utilized for expressing target recombinant proteins. Integration vectors include *E. coli* plasmid replication origin, resistance gene and homologous arms. The feature of restrictive replication means that this type of vector can only be replicated in *E. coli* so that only integration into the host genome will correctly express the target gene. Due to the differences in homologous arms, the integration method can be divided into single and double crossover. The homologous arm needed for single crossover (Fig. 3A) is only one-ended and short, but its integration efficiency is high and stable. On the other hand, in the double exchange method, homologous recombination is achieved through homologous sequences located on both sides of the foreign gene and the target sequence of the host genome (Fig. 3B). In order to improve the integration efficiency of double crossover, effective homologous recombination can be achieved by keeping the length of the homologous arm at least 400–500 bp long. In addition, the stability of linear double chain DNA should also be considered. The commonly used double exchange integration vector is integrated through the *amyE* gene locus, where the inserted foreign gene disrupts the *amyE* gene, and the host is unable to express the amylase, so that positive recombinants can be identified by some strategies. In addition, the thrC gene locus is another choice for inserting a foreign gene into a

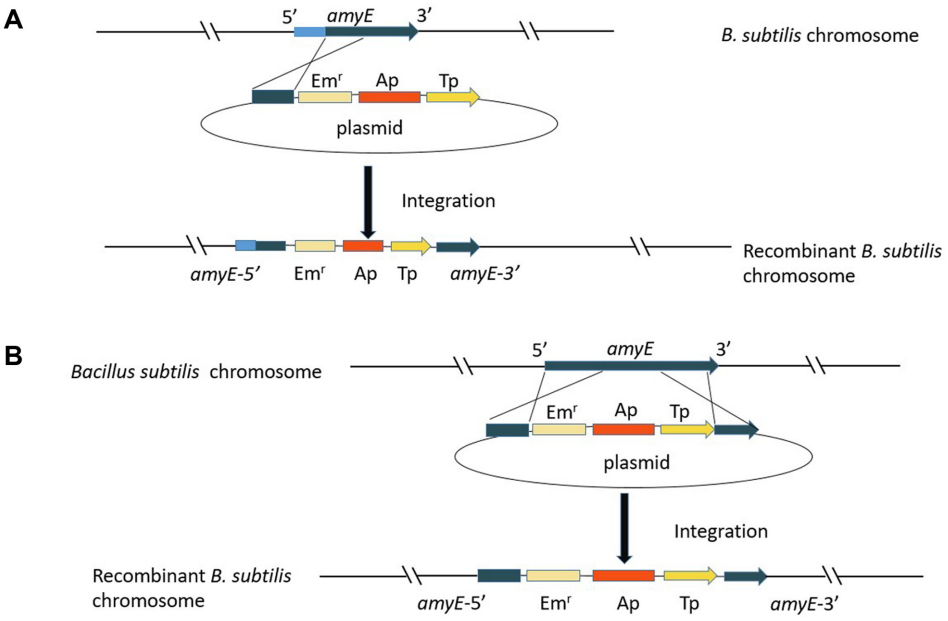

Fig. 3. Methods of integrating target genes into the *B. subtilis* genome: (**A**) single crossover and (**B**) double crossover.

genome, leading to threonine auxotrophy and screening for positive recombinants.

Application of *B. subtilis* Spore Surface Display Technology

BSSD technology is recognized as a powerful technique and is widely used in many fields, including the pharmaceutical and probiotics industries, the food industry and environmental protection. A summary of the applications of this technology as reported by previous studies is given in Table 1.

Preparation of Vaccines

Proteins displayed on the surface of *B. subtilis* spores have unique resistance to harsh conditions and, thus, can smoothly pass through the gastrointestinal barrier. This unique characteristic of spores makes them excellent

Table 1. Expression of various target proteins using *B. subtilis* spore surface display technology and their application in biomanufacturing and bio-controlling.

Target protein	Anchor protein	linker	Fusion method	Application	Reference
TTFC	CotB	NR	C-terminal, N-terminal, and sandwich fusions	Vaccine	[6, 26–28]
Protective antigen	CotB/ CotC	NR	C-terminal	Vaccine	[45]
GST-$Cpa_{247-370}$	CotB	NR	C-terminal	Vaccine	[46]
18×Histidines	CotB	NR	C-terminal	Bio-remediaton	[39]
Urease A	CotB/CotC/CotG	NR	C-terminal	Whole-cell biocatalyst	[47]
Toxins A , B	CotB	NR	C-terminal	Vaccine	[48]
FliD	CotB/CotC/CotG/ CotZ	-GGGEA AAKGGG-	C-terminal	Vaccine	[13]
Envelope protein VP28	CotB	NR	C-terminal	Vaccine	[29]
VP1	CotB	NR	C-terminal	Vaccine	[35]
M2e3	CotB	NR	C-terminal	Vaccine	[36]
SlpA, InvA	CotB	NR	C-terminal	Vaccine	[49]
MPT64	CotB	NR	C-terminal	Vaccine	[50]
a-amylase, GFPuv	CotB/CotC/CotG	-GGGGS-	C-terminal	Functional enzyme	[51]
Urease B, IL-2	CotB/CotC	-GGGEA AAKGGG-	C-terminal	Vaccine	[52]
Esterase	CotB	-GGGGS-	C-terminal	Whole-cell biocatalyst	[53]
Thermophilic lipase Tml350	CotB	-GGGGS-	C-terminal	Whole-cell biocatalyst	[54]
TTFC , LTB	CotC	NR	C-terminal	Vaccine	[55]
CsTP22.3	CotC	NR	C-terminal	Vaccine	[32]
SjGST	CotC	NR	C-terminal	Vaccine	[33]
Bombyx mori Nucleopolyhedrovirus GP64	CotC	NR	C-terminal	Vaccine	
Alcohol dehydrogenases	CotC	NR	C-terminal	Whole-cell biocatalyst	[57]
Human serum albumin	CotC	NR	C-terminal	Oral admini stration	[58]
Proinsulin	CotC	NR	C-terminal	Oral admini stration	[34]
β-Galactosidase	CotC	-GGGGS-	C-terminal	Whole-cell biocatalyst	[59]
Human growth hormone	CotC	NR	C-terminal	Oral admini stration	[60]
Alcohol dehydrogenase A	CotC	NR	C-terminal	Whole-cell biocatalyst	[37]
Enolase	CotC	NR	C-terminal	Vaccine	[31]
Urease B	CotC	NR	C-terminal	Vaccine	[61]
Paramyosin	CotC	NR	C-terminal	Vaccine	[30]
OmpC	CotC	NR	C-terminal	Vaccine	[62]

Table 1. Continued.

Target protein	Anchor protein	Linker	Fusion method	Application	Reference
Streptavidin	CotG	-GGGGS-	C-terminal	Diagnostic tool	[42]
β-Galactosidase	CotG	NR	C-terminal	Whole-cell biocatalyst	[43]
ω-Transaminase	CotG	-GGGGS-	C-terminal	Whole-cell biocatalyst	[41]
N-acetyl-D-neuraminic acid aldolase	CotG	-GGGGS-	C-terminal	Whole-cell biocatalyst	[63]
Hydrolase MfphA, BphD	CotG	-GGGGS-	C-terminal	Whole-cell biocatalyst	[64]
Nitrilase MSB8	CotG	-GGGGS-	C-terminal	Whole-cell biocatalyst	[65]
Chitinase	CotG	NR	C-terminal	Biopesticide	[44]
L-arabinose isomerase	CotG	-GGGGS-	C-terminal	Whole-cell biocatalyst	[12]
Urease A	CotZ/ CotB	-GGGGS-, -GGGEAA AKGGG- (only for CotB)	C-terminal	Vaccine	[23]
D-Psicose 3-Epimerase	CotZ	-GGGEA AAKGGG-	C-terminal	Whole-cell biocatalyst	[14]
Phytase, β-glucuronidase	OxdD/ CotG	-AAAAAA AAAA-	C-terminal	Animal probiotic	[15]
CagA protein	CgeA/CotB/CotC/ CotG/CotZ	-GGGGS- (for CotB, CotC,CotG), -GGGEAAA KGGG- (for CotZ, CgeA)	C-terminal, N-terminal	Vaccine	[20]
β-Glucuro nidase	CotX/CotB/CotC/ CotG	NR	C-terminal	Whole-cell biocatalyst	[16]
β-Glucuro nidase	CotE/ CotG	NR	C-terminal	Whole-cell biocatalyst	[18]
EGFP, β-glucuro nidase	CotY/CotZ	NR	C-terminal	NR	[17]

NR: not reported.

TTFC: the 459-amino-acid C-terminal fragment of the tetanus toxin, GST-$Cpa_{247-370}$: A carboxy-terminal segment of the alpha toxin gene (cpa) fused to the glutathione-S-transferase (GST) gene, toxins A, B: *Clostridium difficile* toxin domains, FliD: *Clostridium difficille* flagellin, VP1: one structure protein Enterovirus 71, M2e3: 3 molcules of M2e consensus sequence of influenza A viruses, SlpA: the S-layer protein from *Lactobacillus brevis*, InvA : Invasion from Yersinia pseudotuberculosis, MPT64: one TB antigen from *Mycobacterium tuberculosis*, LTB: the B subunit of the heat-labile toxin of *Escherichia coli*, CsTP22.3: *Clonorchis sinensis* tegumental protein 22.3 kDa, SjGST: *Schistosoma japonicum* 26 kDa glutathione S-transferase, OmpC: an outer membrane protein (porin) from *Salmonella*.

vehicles for orally administered vaccines. It has been reported that the development of protective antigen proteins or enzymes by using CotB, CotC and CotG as molecular carriers to allow them to be expressed on spore surfaces to construct recombinant spores for immunological or catalytic activity. *B. subtilis* spores, in this regard, can be considered as an attractive platform for oral vaccine production due to their GRAS status. They can serve as low-cost bio-factories and as oral delivery vehicles and do not need complex purification steps, thus providing a low-cost oral vaccine technology.

The spore surface display technique was first applied in the field of oral vaccines. The surface display system was used on bacterial spores by Isticato *et al*. [6] to develop a vaccine precursor that expressed the 459-amino-acid C-terminal fragment of the tetanus toxin (TTFC) with the aid of the CotB fusion partner. This provided the first evidence that an efficient surface expression system using the properties of *B. subtilis* (simple purification, high stability and safety) may be promising for the preparation of bioactive components. Soon afterwards, Duc *et al*. [26], Uyen *et al*. [27] and Ciabattini *et al*. [28] successfully displayed exogenous proteins on TTFC for use as an oral vaccine. These studies demonstrated that spore surface display technology can be used for the production of vaccines and drugs. The VP28 envelope protein of the white spot syndrome virus (WSSV) is considered a candidate antigen for resisting shrimp pathogens (the cause of white spot syndrome, WSS). Nguyen *et al*. [29] targeted the WSSV in shrimp and displayed the VP28 protein of the virus on the spore surface of *B. subtilis*. These shrimp had greatly improved resistance to WSSV infection after being fed recombinant spores. The results of this study suggest that spores expressing VP28 can be used as a

prophylactic treatment antigen for WSS, thereby providing a new type of useful vaccine.

Recently, Sun *et al*. [30] constructed a recombinant plasmid to transform and express *Clonorchis sinensis* paramyosin (CsPmy) on *B. subtilis* spore surfaces. The results of this study showed that oral immunization with this treatment is a safe potential vaccination against clonorchiasis. In a previous study, Wang *et al*. [31] displayed an enolase protein of *C. sinensis* on *B. subtilis* spore surfaces and prepared an oral vaccine by this method. In another study done by Zhou *et al*. [32], *C. sinensis* tegumental protein was expressed (CsTP22.3) on the surfaces of spores to provide protection against *C. sinensis*.

In preparing more effective oral treatments, many researchers have focused on BSSD technology. Li *et al*. [33] showed that the spore surface display system can be applied as a live recombinant vaccine carrier to express *Schistosoma japonicum* glutathione S-transferase (GST). They showed that *B. subtilis* spores could act as promising delivery vehicles to afford more effective vaccination against parasites. Feng *et al*. [34] cloned a foreign gene encoding human proinsulin (HPI) together with the gene encoding CotC to construct a recombinant vector. The strategy of transforming a recombinant vector into *B. subtilis* is double-crossover, and an amylase-inactivated mutant was produced. Oral administration with recombinant spores showed that HPI expressed on spore surfaces could produce a long-term bioactive anti-diabetic drug. Cao *et al*. [35] used *B. subtilis* to display the VP1 protein of EV71 with CotB as a carrier protein to prevent enterovirus 71 (EV71) infection and hand, foot and mouth disease. Zhao *et al*. [36] showed that the CotB-M2e3 fusion protein had no effect on spore structure or function in *B. subtilis* and that heterologous influenza virus M2e3 was successfully expressed on the surface of spores by using CotB as a fusion partner. Importantly, recombinant RSM2e3 spores elicited strong and long-term M2e-specific systemic and mucosal immune responses.

Industrial Enzyme Production

BSSD technology is favorable for expressing enzymes as it offers good stability, improved activity and increased substrate conversion rate. The industrial enzymes expressed on the surface of spores offer the advantages of easy purification and high recovery rates. Guo *et al*. [12] demonstrated that L-arabinose isomerase (L-AI) derived from *Lactobacillus brevis* could be displayed on the surface of *B. subtilis* DB403 spores by using CotG as a fusion partner and linker peptides (GGGGS). The conversion of D-galactose (at a substrate concentration of 125 g/l) to D-tagatose reached 79.7% after 28 h at a productivity rate of 4.3 g/l/h. This L-AI had high specific activity and stability and could be used as a novel whole-cell biocatalyst for D-tagatose production. Another rare sugar, D-Allulose, was explored by He *et al*. [14] for displaying D-Psicose 3-Epimerase (DPEase) anchored at the C-terminus of CotZ via a peptide linker on the surface of *B. subtilis* WB800 spores, and exhibited high thermostability.

β-galactosidase (β–Gal) is another important industrial enzyme with important applications as a whole-cell biocatalyst in the development of dairy and lactulose products. Wang *et al*. [16] developed a new kind of lactulose-producing biocatalyst by displaying β-Gal on the spore surfaces of *B. subtilis* 168. Recombinant CotX-β-Gal was employed to produce lactulose by whole-cell biotransformation, which yielded 8.8 g/l from 200 g/l lactose and 100 g/l fructose.

Despite there being a great deal of literature on BSSD techniques, studies on its applications in food research and technology, such as for the liquor industry, are limited. Alcohol dehydrogenase is the most efficient enzyme used in the liquor industry and converts ethanol into many other substances. Yuan *et al*. [37] successfully displayed *Acetobacter pasteurianus* alcohol dehydrogenase A (adhA) on *B. subtilis* spores by utilizing CotC as a fusion partner to enhance ethanol tolerance. Ethanol tolerance assays indicated that the recombinant bacterial strain provided higher ethanol resistance than that of a wild-type strain.

Controlling Environmental Pollution

B. subtilis spore surface-displayed enzymes are utilized for reducing environmental pollution due to their high stability and catalytic efficiency. Falahati-Pour *et al*. [38] used 1-(3-dimethylaminopropyl)-3-ethylcarbodimide and N-hydroxysuccinimide (EDC-NHS) as cross-linking agents for anchoring organophosphorus hydrolase on spore surfaces. This provided significant improvements in enzymatic activity under various pH and temperature conditions.

Heavy metal pollution of the environment currently poses a great concern. Hinc *et al*. [39] proposed that recombinant spores of *B. subtilis* could be used as a potential bioremediation tool for adsorbing nickel ions. Fusion of CotB and histidine residues was performed to compare the adsorption of nickel by wild-type and recombinant spores and found the latter to be more efficient. Cho *et al*. [40] constructed recombinant vectors, including laccase-encoding gene *cotA*, which were applied to decolorize a widely-used textile dye, indigo carmine, by

over-expressing laccase on the surfaces of *B. subtilis* spores. Experiments indicated that laccase expressed on *B. subtilis* spores may be a promising tool for the treatment of textile dye effluent.

Production of Macromolecular Multimeric Proteins

Spore surface display technology can successfully express large molecular weight and multimeric exogenous proteins on the surfaces of spores without crossing the cell membrane. For example, Hwang *et al*. [41] used spore surface display technology to produce pyridoxal phosphate (PLP) in the presence of CotG, used as an anchoring motif, to display dimers on the surfaces of *B. subtilis* spores. The enzymatic activity of the fusion protein was more than 30 times greater than that of the host spores.

In another study, Kim *et al*. [42] used CotG as an anchor protein for displaying streptavidin on *B. subtilis* spore surfaces. Streptavidin was displayed directly on the spore surfaces during sporulation without crossing the cell membrane. The recombinant spores obtained in this way had good biological activity. Kwon *et al*. [43] successfully displayed another macro-molecular protein, β-galactosidase, on spore surfaces, and found it to be more favorable for maintaining the biological activity of foreign proteins than a vegetative cell-based display system.

Screening of Microecological Preparations

Chitinase can inhibit the growth of many pathogenic fungi that are of great concern to global agriculture. Rostami *et al*. [44] successfully expressed chitinase as a biological control agent on the surfaces of *B. subtilis* spores. In vitro experiments showed that this enzyme can effectively inhibit the growth of *Rhizoctonia solani* and *Trichoderma harzianum* fungi. In another study, Potot *et al*. [15] displayed phytase on the surfaces of spores to prepare animal probiotics. The actions of probiotics depend on the secretion of enzymes needed to maintain proper conformation of active sites in harsh conditions in the gastrointestinal tract, and the strong resistance of spores could provide such resistance.

Future Outlook

BSSD technology has received much research interest in recent years as one of the most effective metabolic engineering approaches. Great progress has been made in food research, for example, to produce new food ingredients like N-acetyl-D-neuraminic acid [66] and rare sugars like D-tagatose [67]. These studies have revealed that spore surface technology could be a promising option for the food industry instead of using the pathogenic host *E. coli*. Furthermore, with the increasing attention on food safety, exploiting target genes and plasmids in probiotic strains like *B. subtilis* for the biosynthesis of food ingredients could hold much promise. However, to achieve this, fundamental research using real-world data aimed at industrial implementation should be encouraged.

Significant progress has been made in this technology, generating broad opportunities in the field of biotechnological manufacturing. Nevertheless, research in some aspects of this technology is still required to further improve it and extend its use in metabolic engineering. One of the first priorities could be the optimization of the gene expression level of anchor proteins by using strong promoters, which could provide a breakthrough. Quynh *et al*. [51] studied IPTG-inducible promoters to regulate the anchor protein CotBΔ105 for the BSSD of α-amylase. However, IPTG is expensive and toxic and, therefore, not suitable for the large-scale fermentation of foreign proteins. Moreover, the intensity of IPTG-inducible promoters is not suitable for high-level expression of exogenous proteins as it is not a tightly controlled promoter. Therefore, comprehensive studies should be encouraged in the future for developing strong promoters through genetic engineering approaches for increased expression efficiency. Moreover, the selection and study of promoters derived from probiotic bacteria or other GRAS bacteria has potential food and medical industry applications.

As a new synthetic device, engineered riboswitches have been demonstrated to be a powerful RNA tool for regulating the expression of protein at the transcriptional and post-transcriptional levels [68]. The construction of artificial riboswitches is mainly based on the assembly of artificial aptamer elements and gene expression regulatory elements, or on the basis of natural riboswitches by directed evolution. To enhance the expression of target genes or inhibit the expression of relevant genes in the target protein synthesis pathway, artificial riboswitches can be incorporated into recombinant plasmids and applied with spore surface display technology to regulate the expression of target proteins on spores.

Over the past several decades, a variety of biological materials, such as enzymes, antibodies, microbial cells and eukaryotic cells, have been extensively used in constructing various biosensors. More specifically, as a good example, *E. coli* cell surface display technology has been applied to construct biosensors [69]. In addition, *B. subtilis* spores also

have been used to construct biosensors [70]. However, to the best of our knowledge, there are no studies on the application of BSSD technology to biosensors. Therefore, there could be great prospects for implementing BSSD technology in biosensors.

Traditional recombinant-related technology for expressing necessary spore surface display proteins has room for improvement in efficiency. This could be enhanced by using more sophisticated techniques, like the modern, fixed-point, gene editing system CRISPR/Cas9, for knocking-in target genes into hosts at the genome level. Over recent years, CRISPR/Cas9 has received much attention in metabolic engineering approaches due to its high efficiency and accuracy. Many previous studies on knock-in [71] or knock-out [72] of key genes has indicated that CRISPR/Cas9 can be utilized in spore surface display technology for the over-expression of target proteins.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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