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MINI-REVIEW

Recent progress in *Bacillus subtilis* spore-surface display: concept, progress, and future

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Abstract With the increased knowledge on spore structure and advances in biotechnology engineering, the newly developed spore-surface display system confers several inherent advantages over other microbial cell-surface display systems including enhanced stability and high safety. *Bacillus subtilis* is the most commonly used *Bacillus* species for spore-surface display. The expression of heterologous antigen or protein on the surface of *B. subtilis* spores has now been practiced for over a decade with noteworthy success. As an update and supplement to other previous reviews, we comprehensively summarize recent studies in the *B. subtilis* spore-surface display technique. We focus on its benefits as well as the critical factors affecting its display efficiency and offer suggestions for the future success of this field.

Keywords Surface display · Spore · *Bacillus subtilis* · Factor · Display efficiency · Application

Introduction

Spore-surface display, which is a powerful technology that can effectively express bioactive molecules on the surface of spores, is becoming increasingly important. In the development of spore-surface display, *Bacillus subtilis* is recognized as the most commonly used *Bacillus* species because of the following: (1) recent advances in knowledge of its spore structure (Driks 1999; Henriques and Moran 2007), (2) amenability and ease of well-established genetic manipulation (Cutting and Vander-Horn 1990), and (3) genomic data (Kunst et al. 1997). Owing to enhanced stability and high safety (Hong et al. 2008; Cutting 2011), the emergence of new recently developed *B. subtilis* spore-surface display that enables the display of various food- or human-related antigens or enzymes on the spore surface for the development of vaccines and whole-cell biocatalysts has helped to advance the field of display methodology (Isticato et al. 2001; Sibley et al. 2014; Kwon et al. 2007; Xu et al. 2011a).

B. subtilis spore display, which possesses exogenous antigen or protein fused to one of the spore coat proteins on the spore surface, celebrates its 15th anniversary in 2016. The initial concept was proposed in 2001 by Isticato and co-workers. Since then, the use of this technology has steadily been progressing due to innovations introduced and more anchoring motifs available. Several excellent recent reviews of independent topics described *Bacillus* spore display (Lee et al. 2003; Wu et al. 2008; Kim and Schumann 2009; Knecht et al. 2011; Pan et al. 2012; Isticato and Ricca 2014), but a comprehensive review of *B. subtilis* spore display is rare. In this review, we highlight new developments in the field of *B. subtilis* spore-surface display. In addition, the prospects of future spore-surface display development are briefly discussed.

Composition of *B. subtilis* spore

In the *Bacillus* genus, the Gram-positive bacterium *B. subtilis* is one of the most widely used species for the production of extracellular enzymes on an industrial scale (Schallmey et al. 2004). When confronted by nutrient depletion, the normally

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rod-shaped *B. subtilis* cells produce a morphologically distinct cell type called the spore. A typical fine structure of the *B. subtilis* spore is shown in Fig. 1. The spore consists of a central core compartment that contains a copy of the chromosome and several layers including proteinaceous spore coat, outer forespore membrane, cortex, and inner forespore membrane. The multilayered spore coat protects the spore against bacterial enzymes, toxic chemicals, and harsh environment. In *B. subtilis*, the coat is made up of a minimum of 70 proteins unique to spores, which comprises four distinct layers: basement layer, inner coat, outer coat, and crust (McKenney et al. 2013) (Fig. 1). The outer forespore membrane, located between coat and cortex, also plays an important role in spore formation (Piggot and Hilbert 2004). The cortex, a thick layer of peptidoglycan, is responsible for maintaining the highly dehydrated state of the core, thereby contributing to the development and maintenance of spore resistance (Imamura et al. 2011). The inner forespore membrane plays a major role in spore resistance to numerous chemicals (Setlow 2006).

Overview of *B. subtilis* spore display

B. subtilis is classified as generally recognized as safe (GRAS) status by the US Food and Drug Administration, and it has been proposed as a probiotic in both human and animal uses (Huang et al. 2008; Hong et al. 2006; Cutting et al. 2009). In addition, *B. subtilis* spores can be easily engineered to display heterologous antigen or protein on their surface. However, such genetically modified *B. subtilis* spores do not affect spore structure and their spore resistant properties under extreme conditions (Hinc et al. 2013). Other advantages include the facts that the fusion protein has not to pass through any membrane barrier and there is no bias toward codon usage. These attributes make *B. subtilis* spore a novel and interesting platform for the display of bioactive molecules. *B. subtilis* spore display is accomplished by inserting a foreign gene encoding an antigen or protein into a *B. subtilis* gene encoding a component of the spore coat. The ideal spore coat protein should only act as an anchor for the displayed antigen and do not interfere with its structure. Moreover, the insertion site of the fusion between the target and anchor protein can affect its stability, activity, and post-translational modification to a certain extent. For minimizing this steric hindrance effect, N-terminal, C-terminal, and sandwich fusions have been introduced. Notably, spore display can provide the target antigen or protein free access to its soluble substrate.

Based on the mechanism of sporulation, a schematic illustrating a possible process for the development of spore-surface display is presented in Fig. 2. The initial step is to transform the recombinant plasmid containing a spore coat protein and heterologous protein fusion gene into the competent *B. subtilis* cells. Then, actively growing cells of recombinant *B. subtilis*

are induced to differentiate into spores. As sporulation proceeds, the rod-shaped cell undergoes an unequal cell division into two compartments, namely, a larger mother cell and a smaller forespore. Subsequently, the *cot* genes encoding spore coat proteins are expressed within the mother cell compartment. Once engulfment is completed, the coat proteins and fusion with coat proteins produced inside the mother cell assemble on the forespore. As a consequence, the forespore is surrounded by two membranes. Eventually, cell lysis occurs in a programmed event to release the mature spore displaying the heterologous protein on its surface into the environment.

Until recently, various microbial cell-surface display systems, such as phage display, *Escherichia coli* cell display, and yeast cell display, have been developed. The advantages and disadvantages of *B. subtilis* spore display and some of the extensive studied surface display systems are illustrated in Table 1. Phage display is the first successful approach for displaying foreign polypeptide on the surface of the phage particle (Smith 1985) and has since been applied for biotechnological and biomedical fields. However, the use of phage display is inherently restricted by the simple post-translation modification and the presentation of smaller functional proteins. Similar to phage display, *E. coli* and yeast cell display systems present their own shortages. The primary disadvantage of the former technology is its low safety, whereas relatively low stability and prolonged fermentation period are the main drawbacks of the latter. For bypassing several problems associated with traditional display systems, *B. subtilis* spore display has been established. Compared with other display technologies, this technique offers additional advantages, such as higher safety and enhanced stability, suggesting that *B. subtilis* spore display is a good molecular display platform to surface express food/human-related proteins or thermolabile enzymes. With the spore-based display system, large and complex enzymes have been expressed on the spore surface. Therefore, *B. subtilis* spore display is a powerful alternative to traditional display systems.

Factors affecting the efficiency of *B. subtilis* spore display

A "one size fits all" surface display system does not exist. The principle and procedure of *B. subtilis* spore display is simple and easy to perform, but the outcome of display efficiency can vary depending upon two elements. One is the amount of expressed protein on the spore surface, and the other is the activity and stability of the displayed protein. More generally, numerous factors affect the efficiency of *B. subtilis* spore display system, including the localization and properties of the anchor protein, the nature of the displayed target protein, how this is linked to the spore coat protein, the type of expression vector and host strain, the use of a flexible peptide linker

Fig. 1 Cartoon structure of a typical *Bacillus subtilis* spore and graphical representation for the surface display of target antigen or protein
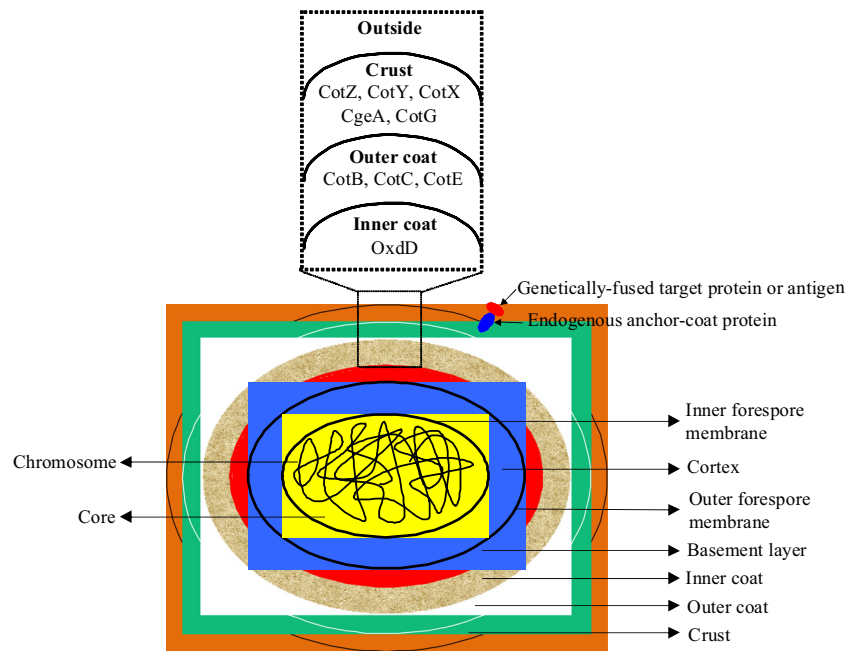

between the spore coat protein and the target protein, and other multiple experimental parameters.

Selection of an appropriate anchor protein is a critical factor in the successful presentation of foreign antigen or protein by *B. subtilis* spore display. The localization and characteristics of the coat protein apparently acts as a vital parameter in candidate anchor protein ranking. Discordance of display efficiency was observed even for the same target protein by using different coat proteins as anchoring motifs. Examples of these proteins are urease subunit A (UreA), flagellar cap protein, α-amylase, GFPuv, and β-galactosidase (Hinc et al. 2010b; Negri et al. 2013; Nguyen and Schumann 2014; Wang et al. 2015, 2016). The size and properties of the displayed target protein are key factors in determining *B. subtilis* spore display efficiency. However, this display technology may tend to fail when applied to the larger and more complex target proteins. Utilizing a high copy number of *E. coli–B. subtilis* shuttle vector, especially non-integrative recombinant plasmid, with the appropriate promoter was shown to exert a beneficial effect on the amount of displayed protein (Xu et al. 2011a; Nguyen and Schumann 2014; Iwanicki et al. 2014; Chen et al. 2015a). In addition, the selection of the appropriate flexible linker inserted into the correct fusion between the anchor protein and the displayed target protein may be essential for stable expression in certain cases (Hinc et al. 2013). Given that the target protein is located on the spore

Fig. 2 Schematic of a possible process for the development of spore-surface display. FX: a fusion of gene encoding coat protein (*blue*) and heterologous protein gene (*red*); FX: a fusion of coat protein (*blue*) linked heterologous protein (*red*)
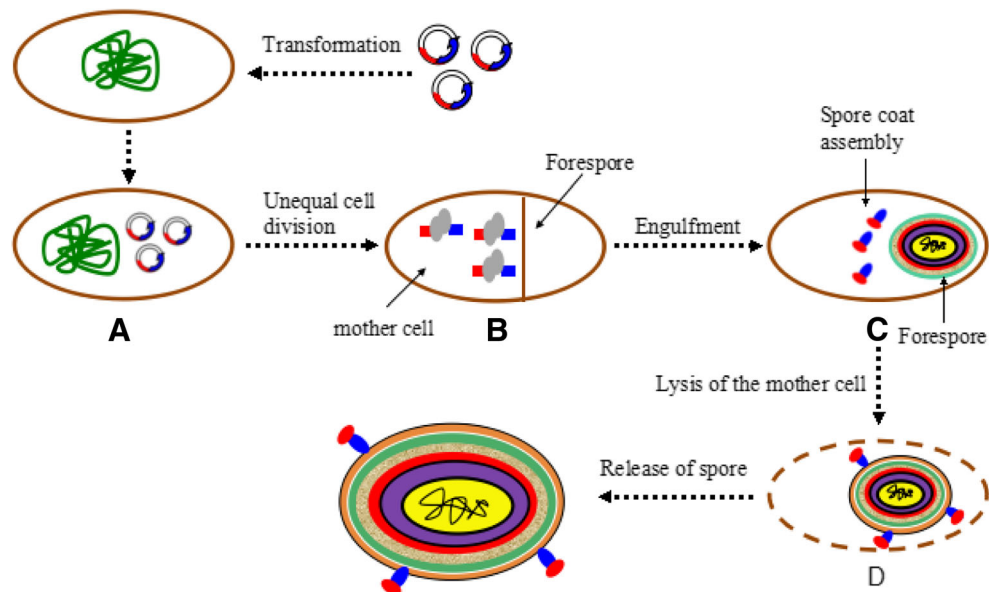
Table 1 A comparison between *Bacillus subtilis* spore display and other microbial cell-surface display systems

	B. subtilis spore display	Other microbial cell-surface display systems		
		Phage display	*E. coli* cell surface display	Yeast cell surface display
Safety	High	Low	Low	High
Stability	High	Low	Low	Low
Post-translation modification	Moderate	Simple	Moderate	Sophisticated
Major field applications	Production of vaccine and biocatalyst	Protein–protein and peptide–DNA interactions, enzyme specificity and inhibitors, antibody, epitopes and mimotopes, receptors and G proteins	Production of biocatalyst, bioadsorption, directed evolution of non-mammalian proteins	Production of biocatalyst, bioadsorption, directed evolution of mammalian proteins

Comparison is according to the cited literature and our laboratory results

surface surrounded by other coat proteins, the surface expression is partially influenced by several abundant and non-fully cooperative spore coat proteins, resulting in lower display efficiency. Deletion of genes encoding the non-fully cooperative coat proteins need to be taken into consideration. Besides the abovementioned factors, the selection of an appropriate host strain is another important factor that affects the display performance of the targeted protein. Cultivation conditions also exert an important influence on the activity and/or the amount of displayed target protein. In general, the display efficiency is extremely challenging to improve, and a careful assessment of numerous factors is required to explore the possible factors that strongly affect display efficiency.

Conventional *B. subtilis* spore display system

The first report on spore-surface display dates from 2001 (Isticato et al. 2001). In the subsequent years, a variety of other spore coat proteins, such as CotC, CotG, CotE, CotX, CotZ, CgeA, and OxdD, have been identified as suitable anchoring motifs that display heterologous antigen or protein on the spore surface of *B. subtilis*. A summary of the published studies on the *B. subtilis* spore-surface display is given in Table 2. Over the past 15 years, when CotB was used as an anchor motif, DNA encoding the three 27 amino acid (aa) repeats located at the C-terminus of CotB was typically omitted, and only DNA encoding the N-terminal 275 aa residues of CotB was used possibly because three repeat fragments cause genetic instability on chimeric proteins containing them (Isticato et al. 2001). In certain cases, however, the full length version of CotB has also been fused to express *Bacillus anthracis* and *Clostridium perfringens* antigens (Duc et al. 2007; Hoang et al. 2008). When all other anchor proteins except CotB were used as fusion partners, DNA encoding the entire Cot proteins

was applied. For obtaining correctly expressed fusion protein, the natural promoter of the *cot* gene is routinely used to control the proper timing of expression of the foreign gene during the sporulation process. The majority of spore display examples often rely on stable integration of an expression construct into the chromosome of *B. subtilis*. However, this approach presents several limitations, such as low expression level and complex operation. Utilizing a high copy number *E. coli–B. subtilis* shuttle vector can help to overcome these problems. As shown in Table 2, CotB and CotC have been successfully used to anchor different antigens on the spore surface for vaccine development, whereas CotG was frequently applied to target functional enzyme display onto the spore for whole-cell catalyst preparation. Until recently, effective antigen presentation by either CotZ or CgeA for identifying potential vaccine candidate has also been reported. CotC has sometimes appeared to be a good candidate for the display of alcohol dehydrogenase (Wang et al. 2011; Yuan et al. 2014) and β-galactosidase (Tavassoli et al. 2013). However, in addition to CotG and CotC, OxdD, CotX, and CotE served as anchoring motifs for proteins such as β-glucuronidase (Potot et al. 2010), L-arabinose isomerase (Liu et al. 2014), or tyrosinase (Hosseini-Abari et al. 2016).

Concerning the conventional *B. subtilis* spore display, the analysis of this approach will be discussed in two aspects according to the number of anchor proteins used.

B. subtilis spore display using an individual spore coat protein as an anchoring motif

CotB

CotB is a 46-kDa protein located in outer layer of *B. subtilis* spore coat. The first evidence that a model antigen C-terminal

Table 2 Summary of *Bacillus subtilis* spore surface-anchored proteins and its related applications

Anchor protein	Linker	Fusion method	Expression system	Target protein or antigen	Application	References
CotB	–	C-terminal, N-terminal, and sandwich fusions	Chromosomal expression	TTFC	Vaccine	Isticato et al. (2001)
	–	C-terminal fusion	Chromosomal expression	TTFC	Vaccine	Duc et al. (2003b); Ciabattini et al. (2004); Uyen et al. (2007); Mauriello et al. (2007)
	–	C-terminal fusion	Chromosomal expression	C-terminal domain of alpha toxin from *Clostridium perfringens as a fusion to glutathione-S-transferase* VP1 of enterovirus 71 VP28 of white spot syndrome virus (WSSV) Ectodomain of influenza virus M2 protein MPT64 from *Mycobacterium tuberculosis* ATCC 27294	Vaccine	Hoang et al. (2008); Cao et al. (2013); Nguyen et al. (2014); Zhao et al. (2014); Sibley et al. (2014)
	–	C-terminal fusion	Chromosomal expression	18 × Histidines	Bioremediation	Hinc et al. (2010a)
	–	C-terminal fusion	Chromosomal expression	A fusion protein streptavidin-CotB bound with biotinylated cetuximab	Anti-cancer drug delivery	Nguyen et al. (2013)
	–	C-terminal fusion	Chromosomal expression	S-layer protein from *Lactobacillus brevis* ATCC 8287 and invasin from *Yersinia pseudotuberculosis* P1 protein of *Streptococcus mutans* NG8	Vaccine	Batista et al. (2014)
	-GGGGS-	C-terminal fusion	Extrachromosomal expression	Lipase Tm1350 from *Thermotoga maritima* MSB8 (ATCC 43589) Esterase from *Clostridium thermocellum*	Whole-cell biocatalyst	Chen et al. (2015a, 2015b)
CotC	–	C-terminal fusion	Chromosomal expression	TTFC and LTB Glutathione *S*-transferase from *Schistosoma japonicum* Envelope glycoprotein GP64 from *Bombyx mori* nucleopolyhedrovirus VP26 of WSSV	Vaccine	Mauriello et al. (2004); Li et al. (2009); Li et al. (2011); Valdez et al. (2014)
	–	C-terminal and N-terminal fusions	Chromosomal expression	TTFC and GFP	Display system	Isticato et al. (2007)
	–	N-terminal fusion	Chromosomal expression		Vaccine	D'Apice et al. (2007)

Table 2 (continued)

Anchor protein	Linker	Fusion method	Expression system	Target protein or antigen	Application	References
	–	C-terminal fusion	Extrachromosomal expression	A T helper cell epitope pep23 from the HIV-1-RT Tegumental proteins TP20.8 and TP22.3 from *Clonorchis sinensis* Enolase and leucine aminopeptidase 2 from *C. sinensis* Urease B from *Helicobacter pylori* Sydney strain 1	Vaccine	Zhou et al. (2008a, 2008b); Wang et al. (2014); Yu et al. (2015); Qu et al. (2014a); Zhou et al. (2015)
	–	C-terminal fusion	Chromosomal expression	Human serum albumin Human proinsulin Human growth hormone	Clinic use	Mao et al. (2012); Feng et al. (2013); Lian et al. (2014)
	–	C-terminal fusion	Chromosomal expression	Alcohol dehydrogenase from *B. mori* Alcohol dehydrogenase A from *Acetobacter pasteurianus* ET-7-3	Whole-cell biocatalyst	Wang et al. (2011); Yuan et al. (2014)
	-GGGGS-	C-terminal fusion	Chromosomal expression	β-Galactosidase from *B. subtilis* 168	Whole-cell biocatalyst	Tavassoli et al. (2013)
CotE	-GGGGS-	C-terminal fusion	Extrachromosomal expression	Tyrosinase from *Bacillis megaterium* DSM319	Whole-cell biocatalyst	Hosseini-Abari et al. (2016)
CotG	-GGGGS-	C-terminal fusion	Extrachromosomal expression	Streptavidin from *Streptomycetes avidinii* ATCC 27419	Diagnostic tool	Kim et al. (2005)
	–	C-terminal fusion	Chromosomal expression	β-galactosidase from *Escherichia coli*	Whole-cell biocatalyst	Kwon et al. (2007)
	-GGGGS-	C-terminal fusion	Extrachromosomal expression	GFPuv	Display system	Kim et al. (2007)
	-GGGGS-	C-terminal fusion	Extrachromosomal expression	*N*-acetyl-D-neuraminic acid aldolase ω-Transaminase from *Vibrio fluvialis* JS17 *meta*-Cleavage product hydrolase Nitrilases from *C. thermocellum* and *T. maritima* MSB8 (ATCC 43589)	Whole-cell biocatalyst	Xu et al. (2011a); Gao et al. (2011); Hwang et al. (2011); Qu et al. (2014b); Chen et al. (2015c, 2016)
	–	C-terminal fusion	Chromosomal expression	Spike protein of transmissible gastroenteritis virus	Vaccine	Mou et al. (2016)
CotX	–	C-terminal fusion	Not provided	β-Galactosidase from *E. coli*	Whole-cell biocatalyst	Kim et al. (2004)
	–	C-terminal fusion	Chromosomal expression	GFP	Display system	Li et al. (2010)
	–	C-terminal fusion	Chromosomal expression	L-arabinose isomerase from *Lactobacillus fermentum* CGMCC 2921	Whole-cell biocatalyst	Liu et al. (2014)
CotG, Oxd-D	-AAAAAAAAAA- (both)	C-terminal fusion	Chromosomal expression	Phytase and β-glucuronidase from *E. coli* K-12	Animal probiotic	Potot et al. (2010)
CotB, CotC	–	C-terminal fusion	Chromosomal expression	Anthrax protective antigen	Vaccine	Duc et al. (2007)
	–	C-terminal fusion		Toxin A peptide repeats	Vaccine	

Table 2 (continued)

Anchor protein	Linker	Fusion method	Expression system	Target protein or antigen	Application	References
			Chromosomal expression	VP28 of WSSV		Permpoonpattana et al. (2011); Colenutt (2014); Ning et al. (2011)
	-GGGEAAAKGGG-(for CotB only)	C-terminal fusion	Chromosomal expression	UreB of *Helicobacter acinonychis* and human IL-2	Vaccine	Hinc et al. (2014); Stasiłojć et al. (2015)
CotE, CotG	–	C-terminal fusion	Chromosomal expression	β-Galactosidase from *E. coli*	Whole-cell biocatalyst	Hwang et al. (2013)
CotB, CotZ	-GGGGS- -GGGEAAAKGGG-(for CotB only)	C-terminal fusion	Chromosomal expression	UreA of *H. acinonychis*	Vaccine	Hinc et al. (2013)
	-GGGEAAAKGGG-(both)	C-terminal fusion	Chromosomal expression	*E. coli* alkaline phosphatase PhoA	Whole-cell biocatalyst	Richter et al. (2015)
CotB, CotC, CotG	–	C-terminal fusion	Chromosomal expression	UreA of *H. acinonychis*	Vaccine	Hinc et al. (2010b)
	-GGGGS- (for CotB only)	C-terminal fusion	Chromosomal expression	α-Amylase Q from *Bacillus amyloliquefaciens* and GFPuv	Display system	Nguyen and Schumann (2014)
CotB, CotC, CotG, CotZ	-GGGEAAAKGGG- (for CotB only)	C-terminal fusion	Chromosomal expression	A fragment of a flagellar cap protein from *Clostridium difficile*	Vaccine	Negri et al. (2013)
CotB, CotC, CotG, CotZ, CgeA	-GGGGS- (for CotB, CotC as well as CotG) -GGGEAAAKGGG- (for CotZ and CgeA)	C- and N-terminal fusions for CotB, CotC and CotG, only C-terminal fusion for CotZ and CgeA	Chromosomal expression	A fragment of cytotoxic-associated protein from *H. pylori* NCTC 11637	Vaccine	Iwanicki et al. (2014)
CotB, CotC, CotG, CotX, CotY, CotZ	–	C-terminal fusion	Chromosomal expression	β-Galactosidase from *Bacillus stearothermophilus* IAM11001	Whole-cell biocatalyst	Wang et al. (2015, 2016)

"–" represents that no linker is inserted between anchor protein and target protein or antigen

fragment of tetanus toxin (TTFC) (Medaglini et al. 2001) can be expressed on the surface of *B. subtilis* PY79 spores by fusion of it to CotB was described by Isticato et al. (2001). In that study, only spores expressing the C-terminal CotB-based fusion protein were able to provide some protection against tetanus in mice. Later on, Duc et al. (2003b) studied mice orally or intranasally immunized with CotB–TTFC. They evaluated the effects on the biological activity (i.e., elicited antitoxin response and the protective efficacy) of these

mice, which displayed *B. subtilis* spores. Unexpectedly, the spore-displayed TTFC can survive transit in the digestive tract. Moreover, a significant protection against a lethal dose of tetanus toxin is also detected in mice orally immunized with recombinant spores.

In 2004, the use of a combined vaccination strategy termed mucosal priming–parenteral boost route to generate strong immune responses against tetanus toxin challenge was explored (Ciabattini et al. 2004). This immunization schedule

resulted in detectable levels of TTFC-specific IgA and IgG responses in both BALB/c and C57BL/6 mice when orally primed with recombinant spores presenting TTFC. Uyen et al. (2007) found that responses against *Clostridium tetani* TTFC (*Cs*TTFC) were enhanced by the use of a combined expression of *Cs*TTFC with spore display and germinating spores. In addition, *Cs*TTFC expressed within the germinating spore provided significantly greater protection against tetanus compared with spore-displayed *Cs*TTFC. Another study revealed that BALB/c mice orally immunized with recombinant spores expressing TTFC on the surface of both germination-defective and congenic wild-type strains stimulated a strong cellular immune response with a Th1 phenotype (Mauriello et al. 2007). In this case, this immune response is independent of spore germination in the animal gastrointestinal tract (GIT). In addition to TTFC, the CotB-mediated spore display was investigated to target other antigens for developing candidate vaccines against necrotic enteritis, enterovirus 71, white spot syndrome virus (WSSV), H1N1 influenza virus, and tuberculosis (Table 2). In these cases, the resulting vaccines were able to elicit antigen-specific immune responses and are protective in animals. All these data clearly demonstrated the use of recombinant spores as a good vaccine vehicle.

In principle, the *B. subtilis* spore-based vaccine can be divided into two classes, namely, recombinant vaccines (spore expressing antigen on its surface) and non-recombinant vaccines (spore-based adsorption of antigen). In both cases, mice immunized with either the recombinant spores or adsorbed spores were demonstrated to be effective and safe in providing protection against infection (Duc and Cutting 2003a; Oggioni et al. 2003; Huang et al. 2010; Permpoonpattana et al. 2011). Sibley et al. (2014) have recently highlighted that both live and formaldehyde-inactivated recombinant spores surface-expressing the MPT64 antigen could elicit Th1 responses in mice, thus proving the effectiveness of the developed TB vaccine against tuberculosis challenge in the murine model. Most importantly, the use of such inactive recombinant spores exerts no ethical consideration associated with the deliberate release of GMOs (Sibley et al. 2014).

Given the extremely short residence time of a recombinant spore-based vaccine in mouse GIT, the oral administration of this type of vaccine is often prone to evoking poor immune responses (Unnikrishnan et al. 2012). This inherent limitation may be overcome by using "gut-colonizing", a modified *B. subtilis* 1012 spore-based, mucosal vaccine delivery system. This system consists of two antigen expression strategies (Batista et al. 2014). The first one contains two different bacterial adhesins, S-layer protein (SlpA) from *Lactobacillus brevis* ATCC 8287 and invasin (InvA) from *Yersinia pseudotuberculosis*. The second one involves P1 protein of *Streptococcus mutans* NG8 as a model antigen. Apart from the presentation of P1 on the spore surface mediated by CotB, one bacterial adhesin was also expressed in germinated

spores. Through this strategy, the expressed bacterial adhesin in the germinated spores facilitates adhesion onto the mucosal surface of animal gut, thereby enabling CotB-P1-displaying recombinant spores to prolong transit through the GIT. Mice immunized by sublingual administration of three doses of *B. subtilis* spores produced higher titers of specific IgG in their serum than mice vaccinated with a ninefold higher dose of spores of the same strains via oral vaccination route (Batista et al. 2014).

Besides its well-known reputation in the development of vaccine vehicles, the CotB-mediated *B. subtilis* spore display has been explored as a potential platform for drug delivery. In cancer therapy, current targeting delivery of anti-cancer drugs to cancerous cells suffers from several drawbacks. The concept of using *B. subtilis* spore display as anti-cancer drug delivery agent was initially developed by Nguyen et al. (2013). For achieving this goal, a clever strategy that relies on a combination of two approaches was applied. One approach is based on the expression of streptavidin as a fusion to the CotB protein at the spore coat, and a second approach involves the adsorption of diterpen paclitaxel, a mitotic inhibitor used for cancer treatment, to the CotB-streptavidin-displaying recombinant spores. By this strategy, the obtained recombinant spores termed "paclitaxel–spore–cetuximab" biocomplex could recognize a primary biotinylated antibody, followed by selective binding to the human epidermal growth factor receptor (EGFR) on HT 29 colon cancer cells, thus enabling the target anti-cancer drug to efficiently suppress cancerous cell division and proliferation (Nguyen et al. 2013). The findings of this study highlight that these recombinant spores displaying streptavidin appear to be an attractive drug delivery system by conjugating with an appropriate biotinylated antibody.

Using CotB as a fusion partner, Chen et al. (2015a) obtained a spore display of a thermophilic, alkali tolerant, and methanol-activated lipase Tm1350 produced by *Thermotoga maritima* MSB8 (ATCC 43589). After spore display, the optimal temperature increased by 10 °C (from 70 to 80 °C) and its optimal pH shifted from 7.5 to 9. Furthermore, the spore-displayed Tm1350 was found to exhibit an increase in thermal stability, pH stability, and reusability compared with those of its free form. In another example, the successful display of a novel thermostable esterase from *Clostridium thermocellum* at the *B. subtilis* spores was achieved with the same anchor protein (Chen et al. 2015b). The resulting spore-displayed esterase not only exhibited a broad temperature and pH optimum but also showed enhanced tolerance to DMSO. The evident improvement in stability of spore-displayed enzymes was probably due to the introduction of a flexible linker (GGGGS) and the use of extrachromosomal expression system from a high copy number plasmid. The overall conclusion of both studies was that the CotB-mediated *B. subtilis* spore display may be a useful tool for enzyme immobilization.

As the above examples demonstrate CotB-mediated spore display for development as vaccine and whole-cell biocatalyst, this approach could also be applied as a bioremediation tool (Hinc et al. 2010a). Spores of *B. subtilis* strain engineered to effectively express 18 histidine residues were demonstrated to be an efficient bioadsorbent for nickel removal. In addition, the efficiency of nickel binding is not influenced by either pH or temperature but is strongly dependent on the amount of spores used in the adsorption reaction (Hinc et al. 2010a).

CotC

CotC (12-kDa) is another spore coat protein in the *B. subtilis*. TTFC has also been displayed as a fusion to CotC as well as the heat-labile enterotoxin B subunit of *E. coli* (LTB); both antigen-displaying spores generated a positive immune response in mice (Mauriello et al. 2004). To improve the display efficiency of the CotC–TTFC fusion protein, both N- and C-terminal fusions were investigated by Isticato et al. in 2007. A fivefold increase in display efficiency was achieved when TTFC was expressed at the N-terminal end of CotC. The effectiveness of the N-terminal fusion approach has also been demonstrated with two T helper cell epitopes pep23 (residues 249–263 of HIV-1 reverse transcriptase) and pep24 (residues 191–205 of HIV-1 gp120) (D'Apice et al. 2007).

Considering both the safety and low-cost production, *B. subtilis* spore display presents a valuable application in vaccine design and delivery. As summarized in Table 2, CotC is mainly used as an anchoring motif for vaccine development. Zhou et al. (2008a, 2008b) searched for an alternative vaccine against clonorchiasis, a disease caused by infection with *Clonorchis sinensis*. They found two putative tegumental proteins (TP20.8 and TP22.3 of *C. sinensis*) that can elicit a significant level of protection in vaccinated rats when delivered as an oral recombinant spore-based vaccine. In recent studies, other enzymes reported to be alternative candidates for the use in vaccine development against *C. sinensis* prevention are enolase of *C. sinensis* (*Cs*enolase) and leucine aminopeptidase 2 of *C. sinensis* (*Cs*LAP2) (Wang et al. 2014; Qu et al. 2014a). The study of Yu et al. (2015) further evidenced that oral immunization in rats with recombinant spores displaying *Cs*enolase induces both systemic and local mucosal immune responses. In addition, other vaccine antigens, including glutathione *S*-transferase of *Schistosoma japonicum*, envelope fusion glycoprotein GP64 of *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV), urease B protein of *Helicobacter pylori* (*Hp*UreB), and VP26 of WSSV were also fused to the C-terminal end of CotC. The immunogenicity of the resulting recombinant spores has been assessed in mouse models in some cases with highly promising results. The reports supporting the use of the CotC-mediated spore display as an ideal vaccine delivery system will continue to grow.

Current interest is focused in utilizing *B. subtilis* spore display as a new delivery vector of therapeutic protein. To date, human serum albumin (HSA), human proinsulin (HPI), and human growth hormone (HGH) have been displayed on the spore surface by using CotC as an anchoring motif. In these cases, the CotC-linked fusion genes were stably integrated into the *B. subtilis* chromosome. The in vivo study demonstrated that mice immunized orally with HSA-expressing spores are capable of increasing blood serum albumin level in a dose-dependent manner (Mao et al. 2012). Although HSA fused to CotC is less efficiently displayed, this spore display format offers several advantages, including high stability and easy production over the yeast cell expression system (Mao et al. 2012). In an alternative approach to the use of mammals for drug development, the same research group studied the application of silkworm for evaluating the therapeutic effects of the recombinant spores expressing HPI or HGH (Feng et al. 2013; Lian et al. 2014). Both studies confirm the efficacy of the recombinant spores that can be digested and absorbed into silkworm hemolymph. The authors reported that this result can presumably be explained by multiple mechanisms, such as the addition of the enterokinase site, inherent resistant properties of *B. subtilis* 168 spores, or the simple intestinal structure of silkworm.

Whereas most of the displayed enzymes tend to be accomplished by fusion to CotG, several recent studies demonstrated that heterologous enzymes on the surface of *B. subtilis* spores can be targeted through the CotC anchoring motif. The first enzyme displayed on *B. subtilis* 168 spores via fusion with CotC was alcohol dehydrogenase from *B. mori* (*Bm*ADH) (Wang et al. 2011). Compared with the native enzyme, the spore-displayed *Bm*ADH exhibits higher enzymatic activity and stability. The β-galactosidase of *B. subtilis* 168 (LacA), a rather large protein of 116 kDa being active as a homotetramer, is another example (Tavassoli et al. 2013). In this study, the gene fusion of *cotC–lacA* was chromosomally integrated into *B. subtilis* 168 and a mutant *B. subtilis* RH101 (CotC-deficient strain), respectively. Western blotting confirmed the expression of the fused *cotC–lacA* at the spore surface of both 168 and RH101 strains. Indeed, both of spore-displayed LacAs could still retain relatively high activities even after exposure to different harsh environmental conditions. An interesting observation in the case of the strain deleted for *cotC* gene is that the CotC–LacA showed better reusability compared with that surface-expressed by *B. subtilis* 168, indicating a higher thermal stability for the spore-displayed LacA. The observed difference may be attributed to the difference in genetic background between 168 and RH101 strains (Tavassoli et al. 2013). A third example is to target alcohol dehydrogenase A from *Acetobacter pasteurianus* ET-7-3 at the spore surface by fusing it with CotC, yielding a modified *B. subtilis* 168 strain with enhanced ethanol tolerance compared with wild-type spores, which

offers an attractive alternative for the liquor industry (Yuan et al. 2014).

CotG

CotG is a 24-kDa protein produced in the outer layer of *B. subtilis* spore coat. Kim et al. (2005) fused the biotin-binding streptavidin to the CotG protein of *B. subtilis* DB104. This protein is the first example of a tetrameric protein anchored on the spore surface. Surface-displayed streptavidin was verified by fluorescence-activated cell sorting and immunological methods. As demonstrated with the CotB-fused anti-cancer drug delivery agent (Nguyen et al. 2013), spore-displayed streptavidin can be a widely used tool in the detection of biotin-conjugated bioactive molecules. GFPuv, one of the most important engineered variants of green fluorescent protein (GFP) (Crameri et al. 1996), along with fusion to CotG, has become an efficient tool for the rapid identification of other possible spore coat proteins in *B. subtilis* (Kim et al. 2007). Another coat protein, CotX, has been proven useful for developing *B. subtilis* spore display and is the result of using this GFPuv as a reporter protein (Li et al. 2011). In a recent work (Mou et al. 2016), transmissible gastroenteritis virus spike (TGEV-S) protein is anchored onto the spores of *B. subtilis* WB800. Following the vaccination of suckling piglets with this recombinant spores, elevated specific SIgA titers in feces, IgG titers, and neutralizing antibodies in serum are generated in response to the in vivo stimulation with TGEV-S antigen. Results show that following TGEV challenge piglets that are passively immunized with the recombinant spores presenting the TGEV-S become highly protected against TGEV via dendritic cells. These findings serve as a valuable basis for developing an appropriate TGEV vaccine by using this spike protein.

For obtaining an enzyme with desirable properties, CotG is often applied as an anchoring motif for the surface expression of native enzymes. The β-galactosidase of *E. coli* (*Ec*β–Gal), the first enzyme to be displayed with CotG, is only stable under mild conditions (Kwon et al. 2007). However, the spore-displayed *Ec*β–Gal not only exhibits an increased tolerance to non-polar hydrophobic organic solvents but could further be stabilized via cross-linking with glutaraldehyde. Furthermore, this spore-displayed *Ec*β–Gal can serve as a new type of biocatalyst to efficiently synthesize octyl-β-D-galactopyranoside from lactose and octanol (Kwon et al. 2007). In addition to *Ec*β–Gal, other enzymes such as *N*-acetyl-D-neuraminic acid (Neu5Ac), ω-transaminase of *Vibrio fluvialis* JS17, *meta*-cleavage product hydrolase (MfphA and BphD) of *Dyella ginsengisoli* LA-4, and nitrilases of both *T. maritima* MSB8 (ATCC 43589) and *C. thermocellum* were also fused to the C-terminus of CotG. According to the results of Xu et al. (2011a), the spore-displayed Neu5Ac aldolase produces a high concentration of Neu5Ac (54.7 g/L) with a yield of 90.2% under optimal conditions. Compared with this new synthetic strategy, virtually the same concentration of Neu5Ac (53.9 g/L) but a lower yield (17.4%) was achieved by a coupled chemoenzymatic process (Gao et al. 2011). Considering the advantages of good stability and easy purification over free or immobilized enzyme, the use of such a spore-displayed Neu5Ac aldolase offers promise for Neu5Ac production. Hwang et al. (2011) reported that after spore display, *V. fluvialis* JS17 ω-transaminase, a cofactor-containing enzyme, exhibited a 30-fold higher enzymatic activity compared with wild-type spores. Another recent research of Qu et al. (2014b) showed that MfphA and BphD from *D. ginsengisoli* LA-4 show increased thermal and pH stabilities as a result of their surface display on spores using CotG as an anchoring motif. Moreover, this spore display not only facilitates the recovery of MfphA and BphD from the reaction mixture but still retains 45 and 70% of their initial activities even after ten consecutive runs, respectively. One of the recent applications of the CotG protein is in the spore display of nitrilase from *T. maritima* MSB8 (ATCC 43589) (Chen et al. 2016). After spore display, the optimal temperature increased by 5 °C (from 45 to 50 °C) and its optimal pH shifted from 7.5 to 8. Besides, spore display induced an increase in thermal stability and pH stability compared with the free form. Furthermore, the spore-displayed nitrilase retained over 80% of initial activity after five successive reaction cycles. A similar result was obtained in case of nitrilase from *C. thermocellum* by the CotG-mediated spore display method (Chen et al. 2015c).

Altogether, the presented examples clearly indicate that among all anchoring motifs reported to date, the CotG-mediated spore display system is excellent for the development of whole-cell biocatalysts.

CotX

CotX has recently been identified as a crust protein (17 kDa) (Imamura et al. 2011). Previously, CotX has been exploited for surface display by fusion to the most utilized reporter protein GFP (Li et al. 2010). Although significant fluorescence could also be detected around the recombinant spores harboring the CotX-linked GFP fusion protein, several advanced analytical methods, such as western blotting and flow cytometry analyses, were not applied to confirm the presence of the protein. Similar to the case of *Ec*β–Gal, no data was available for the verification of the surface expression of *Ec*β–Gal on *B. subtilis* spores (Kim et al. 2004). However, recent studies begin to demonstrate the feasibility of the CotX-mediated spore display. In the study by Liu et al. (2014), L-arabinose isomerase (L-AI) from *Lactobacillus fermentum* CGMCC 2921 was successfully expressed on the surface of *B. subtilis* 168 spores through fusion with CotX. After being displayed, L-AI exhibits a relatively high activity and

improved stability. In addition, a 1.4-fold increase in conversion rate of D-galactose was obtained with this spore-displayed L-AI compared with the previously known system, wherein the highest conversion rate was obtained with the free L-AI (Xu et al. 2011b; Liu et al. 2014). Further evidence of the applicability of the CotX-mediated spore display was found in an example from the β-galactosidase of *Bacillus stearothermophilus* IAM11001 (*Bs*β-Gal) (Wang et al. 2016). After spore display, the optimum temperature shifted from 70 to 75 °C and its pH optimum decreased by 0.5 pH unit (to pH 6.0). Compared with the free *Bs*β–Gal, spore display could also lead to high thermal tolerance and repeated reuse. In addition, with lactose and fructose as substrates, this spore-displayed *Bs*β–Gal produced 8.8 g/L lactulose with a yield of 4.4% (Wang et al. 2016). The results of these two studies validate CotX-mediated spore display as an effective tool in the development of a novel biocatalyst with improved enzyme performance.

OxdD

OxdD is a 43-kDa minor component of the spore coat. Recently, a successfully heterologous surface expression was achieved in *B. subtilis* PY79 by fusion of a monomeric phytase, a commonly used feed enzyme that assists in the breakdown of undigestible phytic acid, to an inner coat protein OxdD (Potot et al. 2010). Spore-displayed phytase was found to increased the nutritional value of feed by liberating the phytase-bound phosphorous, thus lowering the demand for supplementation of inorganic phosphorous. The authors also compared OxdD with CotG to judge whether the former exhibits higher display efficiency for phytase and could be used more frequently for other proteins. As reported, the CotG–phytase fusion protein showed a twofold increase in enzymatic activity compared with fusion to OxdD. This finding led to the proposal that the location of OxdD within the coat may protect the enzyme from the environment but hinder the access of the substrate to the active site of the spore-displayed phytase. Conversely, protection of the displayed enzymes conferred by the OxdD-mediated spore display system may be advantageous in certain applications, especially in the production of orally ingested spore-based probiotic products, as their activities could be well maintained even through the highly acidic and proteolytic environment of the stomach. Moreover, the prospect of OxdD as an anchoring motif was further illustrated by the successful display of a bioactive tetrameric β-glucuronidase from *E. coli* K-12 on the spore surface (Potot et al. 2010).

CotZ

CotZ, a 16-kDa protein required for the assembly of the newly identified spore crust, has been found to act as a new anchoring motif for the efficient display of UreA of *Helicobacter acinonychis* on the spores (Imamura et al. 2011; Hinc et al. 2013). In the case of the CotZ–UreA fusion protein, the calculated number of recombinant protein molecules is 2.5×10^2 from a single spore. This fusion protein is more effective in stimulating immunological response than other antigens in mice. Hinc et al. (2013) attempted to gain insight into the effect of an additional peptide linker on the display efficiency of *H. acinonychis* UreA as a fusion with CotB. They correctly introduced two peptide linkers (GGGGS and GGGEAAAKGGG) between CotB and UreA. However, the CotB–GGGGS–UreA fusion protein failed to be displayed on the spore surface possibly because the inserted short linker cannot form any secondary structure and is thus unable to efficiently stabilize the structure. In contrast, experiments with a longer linker generated promising results, as further demonstrated by western blotting, immunofluorescence microscopy, and dot blot analyses. Furthermore, the expression level of the CotB–GGGEAAAKGGG–UreA fusion protein (1.0×10^4 recombinant molecules per spore) is remarkably higher than that of the CotB–UreA fusion protein without the insertion of an appropriate peptide linker (1.1×10^3) (Hinc et al. 2010b, 2013); this finding states that incorporation of this flexible linker between CotB and UreA resulted in improved display efficiency. Similar results were obtained with FliD when expressed in a same approach for CotB–GGGEAAAKGGG–UreA fusion protein (Negri et al. 2013). This interesting phenomenon may be attributed to the insertion of the flexible longer linker that could form a stable α-helical structure during post-translational processing of the fusion protein.

CotE

In addition to CotG and CotC, another outer coat protein CotE (21 kDa) was used as an anchoring motif for the successful immobilization of *Ec*β–Gal at the surface of *B. subtilis* DB104 spores (Hwang et al. 2013). However, the incorporation of CotE–*Ec*β–Gal fusion protein resulted in disruption of the intact structural integrity of recombinant spore wall, whereas the inserted *Ec*β–Gal fused to CotG exerted no effect on its integrity. This observation provides certain support that the morphogenetic protein CotE is essential to maintain the integrity of the spore wall (Ozin et al. 2001; McKenney and Eichenberger 2012). In addition, the thermal stability of *Ec*β–Gal remained unchanged either for free or for spore-displayed, which is in poor agreement with the previous report (Kwon et al. 2007). Different from CotE–*Ec*β–Gal, CotE–Tyr (a successful spore display of *Bacillus megaterium* DSM319 tyrosinase based on the use of CotE) demonstrated good stability and reusability after spore display (Hosseini-Abari et al. 2016). The insertion of a suitable linker (GGGGS) between CotE and Tyr and the use of extrachromosomal expression

system for Tyr may partially explain this unique catalytic behavior.

CgeA

Similar to CotZ, CgeA is another 14-kDa crust protein, and its possibility in the surface expression of *H. pylori* NCTC 11637 cytotoxic-associated gene A protein (CagA) on the *B. subtilis* 168 spores was investigated (Iwanicki et al. 2014). Both CgeA–CagA and CgeA–GGGEAAAKGGG–CagA fusion proteins were successfully displayed on the surface of *B. subtilis* 168 spores, as confirmed by western blotting and immunofluorescence microscopy analyses. Moreover, the recombinant spores expressing CgeA–linker–CagA exhibited stronger fluorescent signal than recombinant spores displaying CgeA–CagA. When these data are considered together with the data on UreA and FliD, the use of a flexible peptide linker (GGGEAAAKGGG) is suggested to considerably improve the display efficiency of the targeted protein. To further enhance the display efficiency, a universal vector was constructed by introducing an α-helical linker coupled with a selection marker trophic gene (Iwanicki et al. 2014). Given the lack of antibiotic resistance genes in this vector, this approach would offer improved safety in the development of spore-based vaccine or whole-cell biocatalyst (Iwanicki et al. 2014).

B. subtilis spore display using two or more independent coat proteins as anchoring motifs

The inherent benefits of the use of *B. subtilis* spores as antigen delivery platform offer an attractive second-generation vaccine vehicle (Amuguni and Tzipori 2012). When developing an oral vaccine against anthrax caused by the bacterium *B. anthracis*, protective antigen (PA) is often selected because of its stable and highly immunogenic properties. In the study of Duc et al. (2007), the target of PA fragment was displayed on the spore surface by fusing it with CotB or CotC. Full-length PA was also expressed in the germinated spores. Moreover, the highest levels of protective immunity in mice were observed when orally immunized only with recombinant strain, enabling the efficient production of both surface display of PA fragment on the spores and, simultaneously, the secretion of PA from germinating spores. However, the vaccine obtained either with intracellular expression of full-length PA or with N-terminally truncated PA form, which lacks the signal peptide, is unable to provide protective immunity against *B. anthracis* STI anthrax infection (Duc et al. 2007).

Antibiotic-associated diarrhea induced by *C. difficile* has emerged as an important nosocomial infectious. Usually, the major virulence factors of these pathogens are the two toxins A and B (Jank and Aktories 2008). Given the importance of mucosal immunity in *C. difficile* infection, an illustrative

example of how the CotB- and CotC-mediated spore display were used to obtain increased protection in a hamster against *C. difficile* infection when administered orally with the recombinant spores expressing the repeat domain of toxin A has been described previously (Permpoonpattana et al. 2011). Another recent report of Colenutt (2014) provided further evidence on the efficacy of spore display based-vaccine in inducing both systemic and mucosal responses. The delivery of this type of vaccine by a sublingual route resulted in a higher level of protection than that of an oral route. Interestingly, inactivated recombinant spores were more effective than live spores when delivered via the same route.

In addition to toxins A and B, another immunogenic fragment of FliD, the flagellar cap protein of *C. difficile* flagellin, used as a new vaccine candidate has recently been investigated (Negri et al. 2013). By fusion of the fragment of FliD to the C-terminus of CotB, CotC, CotG, or CotZ, successful display of FliD on the surface of *B. subtilis* spores can be achieved; however, in this reported example, the effectiveness of the recombinant spores surface-expressing CotB–GGGEAAAKGGG–FliD, CotC–FliD, CotG–FliD, or CotZ–FliD for developing an oral vaccine against *C. difficile* has yet to be examined in immunization of laboratory animals (Negri et al. 2013). Densitometric analysis shows that the amount of recombinant FliD protein in the spore coat was in the order of CotG–FliD (4.1×10^3 recombinant molecules per spore) > CotC–FliD (3.4×10^3) > CotZ–FliD (2.1×10^3) > CotB–FliD (6.9×10^2). A literature review by McKenney and Eichenberger (2012) suggests that the display efficiency of heterologous antigen or protein on the spore obtained with crust protein may theoretically be considerably higher than that achieved with the outer coat protein of *B. subtilis* spores. However, the results of the CotZ-mediated spore display for the expression of UreA and FliD did not completely support this hypothesis (Hinc et al. 2013; Negri et al. 2013). Apparently, the pattern of localization of fusion proteins within the spore coat may depend mainly on both the nature of target and anchor proteins as well as the characteristics of the fusion protein.

The selection of an adequate antigen could help in developing an efficient anti-*H. pylori* vaccine. Hinc et al. (2010b) attempted to create a vaccine by tethering UreA from *H. acinonychis* on the spores as a fusion to CotB, CotC, and CotG, respectively. Among the tested anchoring motifs, CotB showed the highest display efficiency of UreA. Although both CotC and CotG enabled UreA to be efficiently expressed, no fusion proteins were displayed on the spores. In addition to the failure of spore display of UreA, the CotG-based UreA fusion protein may suffer from partially processed. However, this observation seems to be in poor agreement in the literature on the use of CotG as an anchoring motif for the efficient surface expression of several different proteins, including streptavidin (Kim et al. 2005), β-galactosidase (Kwon et al.

2007), Neu5Ac aldolase (Xu et al. 2011a), and ω-transaminase (Hwang et al. 2011). Recently, another attempt has been made to improve the spore display-based delivery system in terms of immune response (Hinc et al. 2014). As reported, fused to the coat protein CotC, a fragment of subunit B from *H. acinonychis* urease (*Ha*UreBF) was demonstrated to be presented on the surface of *B. subtilis* spores. Apart from the display of *Ha*UreBF, the full-length *ureB* gene of *H. acinonychis* was expressed in vegetative cells. In addition, for the development of a more effective *Ha*UreB vaccine, an appropriate adjuvant, human interleukin 2 (IL-2) displayed with CotB, was used. Results showed that mice immunized via oral route simultaneously with recombinant spores expressing *Ha*UreBF and intracellular expressed *Ha*UreB within vegetative cells developed cellular immune response at a higher level when co-administered with recombinant spores presenting IL-2 compared with mice vaccinated with a vaccine whose production was accomplished through conventional *B. subtilis* spore display (Hinc et al. 2014). Furthermore, the oral administration of recombinant spores presenting *Ha*UreB together with IL-2-displaying spores or with aluminum hydroxide by healthy mice was shown to elicit a mixed Th1/Th17 polarized immune response. This response may provide added protection against this pathogen (Stasiłojć et al. 2015).

WSSV is currently the most serious viral pathogen in crustaceans globally. In a recent research, a highly immunogenic VP28, which is recognized as a promising vaccine candidate against WSSV (Akhila et al. 2014), was displayed on the surface of *B. subtilis* 168 spores by fusion to CotB or CotC (Ning et al. 2011). Oral administration of either recombinant spores displaying CotB–VP28 or CotC–VP28 in invertebrate crayfish generated a significant increase in survival rate, with levels of 37.9 and 44.8%, respectively, compared with 10.3% in crayfish orally immunized with wild-type spores (Ning et al. 2011). Similarly, the survival rate of *Litopenaeus vannamei* vaccinated with the recombinant spores surface-expressing CotB–VP28 was significantly higher than that of untreated animals after 14 days (Nguyen et al. 2014). Therefore, the administration of the recombinant spores displaying VP28 could strengthen the immune abilities of both crayfish and *L. vannamei* and increase their resistance to WSSV infection.

As previously noted, optimization of the promoter sequence is another approach to improve spore display efficiency. In a recent work done by Nguyen and Schumann (2014), two different β-D-thiogalactoside (IPTG)-inducible promoters P_{grac} and P_{Sgrac} were tested for the respective surface expression of α-amylase Q (AmyQ) from *Bacillus amyloliquefaciens* and GFPuv on the spore using CotB, CotC, and CotG as anchoring motifs. The P_{grac} promoter was found to enable the display of both AmyQ and GFP$_{uv}$ on the spore surface. The expression level of CotB–AmyQ displayed on the spore coat under control of P_{Sgrac} was the highest as compared to expression levels driven by P_{grac} or P_{cotB}, but their activities followed the order of P_{grac}–CotB–AmyQ > P_{cotB}–CotB–AmyQ > P_{Sgrac}–CotB–AmyQ, postulating the existence of an enzymatically inactive form of AmyQ on the spore surface. Furthermore, the authors observed that only overproduced proteins among the three utilized coat proteins for displaying GFPuv tend to accumulate at different positions on the spore surface (Nguyen and Schumann 2014). The difference in arrangements may be a result of self-interaction within each overproduced coat protein and their tendency of localization on the spore coat.

More recently, by fusion of *Bs*β–Gal to the C-terminus of each of CotB, CotC, CotG, CotX, CotY, or CotZ, an active enzyme could be displayed on the spore surface of *B. subtilis* 168 (Wang et al. 2015, 2016). The activity of spore-displayed *Bs*β–Gal was in a sequence of CotG–*Bs*β–Gal > CotX–*Bs*β–Gal > CotB–*Bs*β–Gal > CotZ–*Bs*β–Gal > CotC–*Bs*β–Gal > CotY–*Bs*β–Gal. In addition, CotX–*Bs*β–Gal was further tested for its transgalactosylation activity, yielding 8.8 g/L of lactulose from lactose and fructose. Hence, similar to CotG, CotX can exhibit considerable potential to target more bioactive molecules at the surface of *B. subtilis* spores. PhoA, an alkaline phosphatase of *E. coli*, is a disulfide bond-containing protein and becomes biologically active only when disulfide bonds can be formed. As shown by Richter et al. (2015), PhoA was correctly anchored on the *B. subtilis* spores as a fusion with either CotB or CotZ. The activity of spore-displayed PhoA was confirmed by enzyme assay. Further, analysis of the double-knockout experiment revealed that the deficient in both two proteins BdbC and BdbD exert no effect on the formation of disulfide bonds in recombinant spores surface-expressing PhoA. Collectively, these data prove that *Bacillus* spore display is a more suitable method for the immobilization of these disulfide-bonded-containing proteins.

Innovative *B. subtilis* spore display system

In all conventional *B. subtilis* spore display formats discussed so far, the system requires an anchor protein to target the displayable antigen or protein onto the spore surface. As stated above, this display format also presents certain limitations. However, researchers have been and have continue to invest considerable effort toward increasing *B. subtilis* spore display efficiency. With considerable increase in knowledge on spore structure and recent advances in biotechnology engineering, an innovative approach for direct display of native protein on *B. subtilis* spore without anchor protein as a scaffold was first proposed by Pan et al. (2014). In that report, the key element in determining the success of this native protein display system is the *cry1Aa* promoter, which is a σ^E- and σ^K-dependent promoter. When the gene of interest is placed under the

sporulation-specific promoter, the target protein is preferably synthesized and displayed onto the spore surface. Through the use of this novel display strategy, two model enzymes, one being monomeric carboxymethylcellulase (CMCase) and the other being tetrameric β-galactosidase, can be successfully displayed on the spore surface in their respective native form, as verified by enzyme activity assays, flow cytometry, and immunogold electron microscopy experiments. Despite the demonstrated success in both CMCase and β-galactosidase, the general applicability of this promising display system along with the use of *cry1Aa* promoter for other proteins needs further investigation. As compared to non-recombinant spore display, the innovative *B. subtilis* spore display system offers clear advantages, namely, obviating the need for protein purification and preventing undesired detachment of displayed protein from the spore surface. This innovative *B. subtilis* spore display will not only widen the range of displayable proteins but also expand application fields.

Conclusions and future prospects

This review has highlighted several of the recent progress in understanding *B. subtilis* spore display. As a powerful tool, *B. subtilis* spore display conceivably offers broad possibilities in its future. However, current application of *B. subtilis* spore display is not yet feasible from laboratory-scale trial to commercialization. The very low display efficiency is a main barrier that blocks the development of *B. subtilis* spore display. Further studies on the exploration of a wider variety of anchor protein present a promising approach to address this great challenge. In addition, the display performance of heterologous antigen or protein can be potentially increased by approaches such as redesigning the surface structure of spore coat, optimization of coat protein folding and assembly, directed evolution of coat protein, increasing the copy number of the gene cassettes, and construction of a versatile vector. Meanwhile, the excellent availability of genetic manipulation tool for *B. subtilis* will help in the redesign and exploitation of metabolic pathways for improved target antigen or protein expression in display host cells. Importantly, several issues involved in innovative *B. subtilis* spore display system should also be addressed. For instance, how does this native protein display work, what about display efficiency when compared with the conventional display format, and can the display serve as drug delivery vehicle and withstand in harsh conditions?

However, application aspects of *B. subtilis* spore display will require additional directions. One possibility is to catalyze multi-step transformation for directly producing bio-based chemicals by applying *B. subtilis* spores that co-display a combination of the appropriate enzymes. An exciting field is in the targeted drug delivery system for therapy in diverse diseases. Another approach is devoted to the continuing development of more effective vaccine with unique properties. In view of the past innovation in the field, *B. subtilis* spore display tool is expected to be commercialized for industrial applications.

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