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Applications of Surface Display of Exogenous Functional Proteins on Bacillus subtilis Spores (Postprint)

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Abstract

Bacillus subtilis is an aerobic probiotic strain that can be directly used in humans and animals and can be induced to form spores under adverse conditions. Spores possess a special structure and unique physiological characteristics. Researchers have discovered that Bacillus subtilis spores serve as ideal anchoring vehicles for exogenous functional proteins such as enzymes and immunogens. By employing Bacillus subtilis spore coat proteins as molecular carriers, exogenous proteins are anchored onto the spore surface through methods including direct spore adsorption and covalent immobilization. To date, a variety of enzyme proteins, antigen proteins, and other functional proteins have been successfully displayed on the surface of Bacillus subtilis spores. This article primarily elaborates on the structure of Bacillus subtilis spores, as well as the strategies and application prospects of spore surface display technology for exogenous proteins.

Full Text

Application of Bacillus subtilis Spore Surface Display Technology for Heterologous Functional Proteins

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Abstract

Bacillus subtilis is an aerobic Gram-positive bacterium widely used as a probiotic for humans and animals that can be induced to form spores under adverse conditions. Due to their unique structure and physiological characteristics, *B. subtilis* spores have been identified as ideal anchoring vehicles for heterologous functional proteins such as enzymes and immunogens. Using spore coat proteins as molecular carriers, foreign proteins can be displayed on the spore surface through direct adsorption or covalent immobilization. To date, numerous enzymes, antigens, and other functional proteins have been successfully displayed on the surface of *B. subtilis* spores. This review primarily elaborates on the structure of *B. subtilis* spores, strategies for spore surface display technology, and its application prospects.

Keywords: *Bacillus subtilis*; spore surface display; covalent immobilization; vector

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Bacillus subtilis is a spore-forming Gram-positive bacterium that has attracted considerable research attention due to its probiotic properties and the robust stress resistance of its spores. Spore surface display technology exploits the unique architecture of spores to anchor heterologous functional proteins on their surface through specific strategies, thereby enhancing protein functionality and stability. Isticato et al. [1] first demonstrated this approach by using the spore coat protein CotB as an anchor to successfully display the tetanus toxin C fragment (TTFC) on the spore surface. Characterized by simple operation and high stability of recombinant spores, this technology has found successful applications in diverse fields including mucosal vaccines, biocatalysis, biodegradation, and diagnostic tools. The technology has evolved into two distinct anchoring strategies: genetic recombination and non-genetic recombination [2]. The genetic recombination approach employs spore coat proteins such as CotB, CotC, CotE, CotG, CotZ, and OxdD as anchors, fusing heterologous proteins with these coat proteins for surface display [1,3-7]. In contrast, the non-genetic recombination approach relies on adsorption between spores and heterologous proteins or uses cross-linking agents like glutaraldehyde to immobilize proteins on the spore surface [8-9].

1.1 Sporulation

Under adverse conditions, *Bacillus subtilis* undergoes a series of temporally and spatially regulated gene expression events, ultimately forming a dormant structure—the spore—to withstand harsh environments. Sporulation in *B. subtilis* vegetative cells is intimately associated with a complex network of feedback and feedforward reactions [10]. Environmental signals activate internal phosphorylation cascades that influence the phosphorylation level of the key transcriptional regulator Spo0A, thereby controlling the transition from vegetative growth to sporulation. Low-level phosphorylation of Spo0A promotes biofilm formation,

whereas high-level phosphorylation initiates the sporulation process [11]. Once the bacterium commits to sporulation, it undergoes a time-consuming, energy-intensive, and irreversible process that culminates in spore formation at the progeny level.

The sporulation process can be broadly divided into several stages [10]. First, vegetative cells initiate sporulation in response to environmental stress, with replicated chromosomes anchored at opposite cell poles via the RacA protein. Subsequently, a septum divides the cell asymmetrically into a forespore and a mother cell, activating F and E factors in the forespore and mother cell, respectively. These activated sigma factors regulate the expression of relevant genes, enabling the mother cell to "engulf" the forespore, resulting in a structure enveloped by two membranes—the bacterial cell wall and a thin peptidoglycan layer—while also activating G and K factors in both compartments. The mother cell orchestrates the synthesis and assembly of the forespore cortex and spore coat layers, and finally lyses through the action of autolysins to release the mature spore.

1.2 Spore Structure

The spore is a highly stress-resistant dormant structure that exhibits exceptional tolerance to radiation, high temperature, and chemical agents. *Bacillus subtilis* spores consist of three main layers from inside to outside: the core, cortex, and spore coat. The core contains the bacterial genome DNA and associated proteins, with extremely low water content. The removed water is replaced by 2,6-pyridinedicarboxylic acid (DPA), and the genomic DNA is complexed with / -type small acid-soluble spore proteins (SASPs).

SASPs play a crucial role in protecting dormant spores from environmental insults such as moist heat, desiccation, and hydrogen peroxide that could damage genomic DNA, thereby preventing spore death [12]. DPA is essential for UV resistance, maintaining core dehydration, preserving spore dormancy, and facilitating germination [13-14]. The cortex comprises a specialized peptidoglycan layer located between the inner and outer forespore membranes, conferring resistance to high temperature and desiccation. The spore coat consists of four proteinaceous layers from inner to outer: the basement layer, inner coat, outer coat, and crust, comprising over 70 different proteins that constitute the first barrier of the spore. Coat assembly is believed to initiate through the interaction of SpoVM protein with the outer forespore membrane surface. SpoVM is a 26-amino-acid amphipathic -helical protein less than 4 nm in length that anchors SpoIVA protein to the outer forespore membrane surface through interactions with both SpoIVA and the membrane itself [15]. Ramamurthi et al. [16-17] demonstrated that the length and structure of SpoVM, along with the convex curvature of the outer forespore membrane, are critical for guiding the proper localization of SpoIVA on the membrane surface.

Spore morphogenetic proteins are a class of proteins that play essential roles

in the assembly of spore layers without affecting gene expression in the mother cell. Proper coat assembly heavily relies on the correct expression of these morphogenetic proteins. For instance, in SpoIVA mutants, coat proteins can assemble around the forespore but remain as free-floating layers rather than being anchored to the outer forespore membrane. In SpoVM mutants, the coat is only partially and disorganizedly anchored to the forespore surface. Additionally, *safA* and *cotE* are required for the assembly of inner and outer coat layers, respectively [18].

2.1 Genetic Recombination

The genetic recombination strategy for spore surface display of heterologous proteins is primarily based on gene fusion between foreign DNA and coat protein DNA. In engineered bacteria, the heterologous protein is co-expressed with the coat protein during spore formation, and the resulting fusion protein is ultimately anchored on the spore surface via the coat protein without compromising spore structure or function [19]. Based on the recombination approach, spore surface display strategies can be categorized into free-type and integration-type systems. The free-type system involves introducing a recombinant plasmid carrying the fusion gene into the host for replication and expression, whereas the integration-type system incorporates homology arms flanking the fusion gene, enabling linearized plasmid vectors to integrate the fusion gene into the host genome through double-crossover recombination for subsequent replication and expression. While the free-type approach is operationally simple, it suffers from plasmid instability, whereas the integration-type system offers superior genetic stability.

Plasmid vectors employed in free-type spore surface display are predominantly chimeric constructs derived from *Bacillus* plasmids and *Escherichia coli* pBR322 or its derivatives, such as pHP13, pHPS9, pCSK1, and pLJ7 [20-21]. Most free-type plasmid vectors utilize coat protein promoters to drive fusion protein expression in the shuttle plasmid, which becomes activated during sporulation to express the fusion protein that is ultimately anchored on the spore surface. Nguyen et al. [22] reported that using IPTG-inducible promoters in shuttle plasmids pQAS34 and pQAS32, and adding the inducer IPTG at the t2 stage of sporulation, resulted in significantly higher levels of target protein detected on the spore surface compared to strains relying solely on native coat protein promoters.

Integration vectors are fundamentally composed of a non-replicative plasmid backbone derived from *E. coli* pBR322 or its derivatives, a selectable marker gene for *B. subtilis*, and a DNA fragment homologous to the *B. subtilis* chromosome. The homologous DNA fragments typically target non-essential genes such as *amyE*, *thrC*, *lacA*, *pyrD*, *gltA*, and *scaA* as integration sites [21], with the resistance gene inserted between the homology arms. These arms also contain one or multiple restriction sites for introducing foreign fragments. Since these plasmids lack a *B. subtilis* replication origin, they cannot replicate au-

tonomously after transformation and must integrate into the host chromosome to be maintained through cell division. The integrated fusion gene is subsequently expressed under the control of a coat protein promoter and anchored on the spore surface.

2.2 Non-Genetic Recombination

The non-genetic recombination strategy for spore surface display involves incubating spores with purified heterologous proteins. Through electrostatic and hydrophobic interactions between spores and proteins, the heterologous proteins become adsorbed onto the spore surface [23]. Donadio et al. [24] proposed that during incubation, heterologous proteins may permeate through the spore cortex and outer coat layers to interact with inner coat proteins, thereby achieving more stable anchoring. More recently, methods employing cross-linking agents such as glutaraldehyde have been developed, which utilize covalent bonds formed between glutaraldehyde, spores, and heterologous proteins to immobilize the proteins on the spore surface [9]. This non-genetic approach not only eliminates the need for specific coat proteins but also enables higher loading of target proteins on the spore surface compared to genetic recombination methods, while avoiding the introduction of resistance genes into the environment.

3 Applications of Spore Surface Display Technology

Bacillus subtilis possesses well-established fermentation protocols and production technologies, and its spores exhibit exceptional stress resistance, rendering spore surface display technology applicable across a broad spectrum of fields. Currently, *B. subtilis* spore surface display technology has been extensively employed in the production of mucosal vaccines, vaccine adjuvants, polymeric proteins, bioremediation agents, and feed enzymes.

3.1 Applications in Mucosal Vaccine Production

Bacillus subtilis is a well-recognized probiotic bacterium whose spores possess remarkable stress resistance, enabling them to survive gastric transit and reach the intestine where they are taken up by M cells and interact with antigen-presenting cells in Peyer' s patches [25-26]. This property confers unique advantages for *B. subtilis* as a mucosal vaccine delivery vehicle or adjuvant. Additionally, recombinant spores offer other benefits including relatively low storage and transportation requirements, simple administration routes for animal immunization, and reduced stress during the vaccination process.

Duc et al. [27] immunized mice orally and intranasally with recombinant spores of strain RH103 displaying TTFC, successfully inducing specific secretory IgA (sIgA), IgM, and Th2-biased immune responses dominated by IgG1 and IgG2b. These results demonstrate that recombinant spore immunization can elicit both local mucosal and systemic humoral immune responses. Challenge experiments

showed that mice immunized via oral or intranasal routes could survive subcutaneous challenge with 20 times the lethal dose (LD) of tetanus toxin. Zhou et al. [3] displayed *Helicobacter pylori* urease on the spore surface using CotC as an anchor and the free-type plasmid pUS186 as a vector. Oral administration of the recombinant spores stimulated mucosal and humoral immune responses, and challenge experiments after three immunizations demonstrated an 84% reduction in gastric *H. pylori* colonization. Liu Minggang et al. [28] from our laboratory displayed the *Salmonella pullorum* outer membrane protein C gene (*OmpC*) using CotB as an anchor, providing 100% and 50% protection against *Salmonella typhimurium* challenge at 2× and 10× LD doses, respectively. Notably, administration at a typical probiotic dosage of 1×10 CFU/g in feed produced superior intestinal mucosal and serum antibody responses. Huang et al. [8] anchored the GST-Cpa247–370 fusion protein on spore surfaces through adsorption, achieving comparable antibody levels via both oral and intranasal immunization routes and conferring protection against challenge with 6× LD of toxin. Manki et al. [29] immunized mice intranasally with inactivated spores adsorbed with inactivated H5N1 viral particles, resulting in earlier detection and higher levels of specific antibodies. Aps et al. [30-31] further demonstrated that spores can serve as DNA vaccine adjuvants; mice immunized with spores adsorbed with plasmid DNA vaccines showed enhanced specific antibody levels and increased CD8 T cell activation.

3.2 Applications in Polymeric Protein Production

The unique sporulation process of *B. subtilis* confers a distinctive display mechanism for heterologous proteins in recombinant spores. Heterologous proteins synthesized in the mother cell can be directly assembled on the spore surface without transmembrane transport, offering particular advantages for producing proteins that require multimeric assembly for activity. Immobilizing enzymes on spore surfaces through spore surface display technology not only simplifies enzyme purification and recovery but also enhances enzyme activity and stability. Kim et al. [32] successfully displayed functionally active streptavidin on *B. subtilis* spore surfaces, demonstrating that multimeric proteins can be correctly assembled on spores. Richter et al. [33] found that disulfide bonds in alkaline phosphatase displayed on spore surfaces could form spontaneously. Sirec et al. [34] displayed -galactosidase on spore surfaces via adsorption, significantly increasing the enzyme' s tolerance to acidic conditions (pH 4) and high temperatures (75–80 °C). The successful anchoring of active multimeric proteins on spore surfaces validates the applicability of this technology for polymeric protein production, providing a novel alternative for manufacturing such proteins.

3.3 Applications in Environmental Bioremediation

With advances in microbial remediation of environmental pollution, biocatalysis employing enzymes has been increasingly applied to environmental management due to its eco-friendliness and high catalytic efficiency. Chen et al. [4]

anchored nitrilase—an important industrial enzyme—on spore surfaces, simplifying enzyme purification and recovery while enhancing tolerance to temperature, pH, and chemical agents. Improved enzyme tolerance is crucial for maintaining high catalytic efficiency under the complex conditions encountered in industrial wastewater treatment.

Falahati-Pour et al. [35] used 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide (EDC-NHS) as cross-linking agents to covalently immobilize organophosphorus hydrolase on spore surfaces. This immobilization method significantly enhanced enzyme activity across various pH and temperature conditions. After six cycles of reuse, enzyme activity remained nearly unchanged, and even after ten cycles, 80% activity was retained. This approach provides a novel bioremediation strategy for addressing organophosphorus pesticide residues in the environment.

3.4 Microecological Preparations with Specific Functions

Spore surface display technology enables the construction of strains with specific functionalities, overcoming limitations of traditional probiotics such as complex screening processes, single effects, and poor targeting. This technology has been explored for producing feed enzyme preparations. Potot et al. [7] displayed microbial phytase on spore surfaces, and the inherent stability of spores conferred enhanced enzyme stability. This approach holds promise for addressing issues in conventional feed enzyme production, including inconsistent solid-state fermentation quality, low enzyme yields, and activity loss during feed pelleting and storage. Feng Fan [36] further engineered a fusion protein by inserting an enterokinase cleavage site between human proinsulin and the anchor protein. When recombinant spores were fed to silkworms, human proinsulin could be detected in the hemolymph, demonstrating that spore-displayed recombinant proteins can be digested by enterokinase and absorbed into the bloodstream.

Bacillus subtilis spore surface display technology has evolved multiple display strategies that transform spores into versatile vehicles. Spores exhibit exceptional stress resistance, require simple storage conditions, can traverse the gastrointestinal barrier, and interact with antigen-presenting cells. Moreover, *B. subtilis* itself is a probiotic that modulates intestinal microbiota and enhances immunity. These attributes collectively establish *B. subtilis* spores as excellent mucosal vaccine delivery vehicles and immune adjuvants. Recent discoveries have further revealed that spores can serve as DNA vaccine carriers, suggesting that spore surface display technology will provide novel avenues for vaccine design. Beyond enhancing vaccine efficacy and improving heterologous protein function, *B. subtilis* can exert microecological effects, offering new directions for developing microecological preparations with specific functions. Spore surface display technology enables direct enzyme immobilization on spore surfaces, yielding enzymes with enhanced stability and simplified purification and recovery processes, thus providing innovative options for immobilization carriers in the biocatalysis industry. The construction of multifunctional recombinant

spores using two anchor proteins or different display strategies will further establish spores as multifunctional platforms. With continued research advances, *B. subtilis* spore surface display technology is poised to play increasingly important roles across diverse fields.

References

[1] Isticato R, Cangiano G, Tran HT, et al. Surface display of recombinant proteins on *Bacillus subtilis* spores[J]. Journal of Bacteriology, 2001, 183(21): 6294-6301.

[2] Isticato R, Ricca E. Spore surface display[J]. Microbiology Spectrum, 2014, 2(5): 351-366.

[3] Zhou Z, Gong S, Li XM, et al. Expression of *Helicobacter pylori* urease B on the surface of *Bacillus subtilis* spores[J]. Journal of Medical Microbiology, 2015, 64(1): 104-110.

[4] Chen H, Chen Z, Ni Z, et al. Display of *Thermotoga maritima* MSB8 nitrilase on the spore surface of *Bacillus subtilis* using outer coat protein CotG as the fusion partner[J]. Journal of Molecular Catalysis B: Enzymatic, 2016, 123: 73-80.

[5] Hinc K, Iwanicki A, Obuchowski M. New stable anchor protein and peptide linker suitable for successful spore surface display in *B. subtilis*[J]. Microbial Cell Factories, 2013, 12(1): 22.

[6] Hosseini-Abari A, Kim BG, Lee SH, et al. Surface display of bacterial tyrosinase on spores of *Bacillus subtilis* using CotE as an anchor protein[J]. Journal of Basic Microbiology, 2016, 56(12): 1331-1337.

[7] Potot S, Serra CR, Henriques AO, et al. Display of recombinant proteins on *Bacillus subtilis* spores, using a coat-associated enzyme as the carrier[J]. Applied and Environmental Microbiology, 2010, 76(17): 5926-5933.

[8] Huang JM, Hong HA, Van Tong H, et al. Mucosal delivery of antigens using adsorption to bacterial spores[J]. Vaccine, 2010, 28(4): 1021-1030.

[9] Ghaedmohammadi S, Rigi G, Zadmard R, et al. Immobilization of bioactive protein A from *Staphylococcus aureus* (SpA) on the surface of *Bacillus subtilis* spores[J]. Molecular Biotechnology, 2015, 57(8): 756-766.

[10] Tan IS, Ramamurthi KS. Spore formation in *Bacillus subtilis*[J]. Environmental Microbiology Reports, 2014, 6(3): 212-225.

[11] Higgins D, Dworkin J. Recent progress in *Bacillus subtilis* sporulation[J]. FEMS Microbiology Reviews, 2012, 36(1): 131-148.

[12] Setlow P. I will survive: DNA protection in bacterial spores[J]. Trends in Microbiology, 2007, 15(4): 172-180.

[13] Slieman TA, Nicholson WL. Role of dipicolinic acid in survival of *Bacillus subtilis* spores exposed to artificial and solar UV radiation[J]. Applied and Environmental Microbiology, 2001, 67(3): 1274-1279.

[14] Magge A, Granger AC, Wahome PG, et al. Role of dipicolinic acid in the germination, stability, and viability of spores of *Bacillus subtilis*[J]. Journal of Bacteriology, 2008, 190(14): 4798-4807.

[15] Gill RL, Casting JP, Hsin J, et al. Structural basis for the geometry-driven localization of a small protein[J]. Proceedings of the National Academy of Sciences, 2015, 112(15): E1908-E1915.

[16] Ramamurthi KS, Clapham KR, Losick R. Peptide anchoring spore coat assembly to the outer forespore membrane of *Bacillus subtilis*[J]. Molecular Microbiology, 2006, 62(6): 1547-1557.

[17] Ramamurthi KS, Lecuyer S, Stone HA, et al. Geometric cue for protein localization in a bacterium[J]. Science, 2009, 323(5919): 1354-1357.

[18] McKenney PT, Driks A, Eichenberger P. The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat[J]. Nature Reviews Microbiology, 2013, 11(1): 33-44.

[19] Rosales-Mendoza S, Angulo C. *Bacillus subtilis* comes of age as a vaccine production host and delivery vehicle[J]. Expert Review of Vaccines, 2015, 14(8): 1135-1148.

[20] Schumann W. Production of recombinant proteins in *Bacillus subtilis*[J]. Advances in Applied Microbiology, 2007, 62: 137-189.

[21] Yang MM, Zhang WW, Zhang XF, et al. Construction and characterization of a novel maltose-inducible expression vector in *Bacillus subtilis*[J]. Biotechnology Letters, 2006, 28(21): 1713-1718.

[22] Nguyen QA, Schumann W. Use of IPTG-inducible promoters for anchoring recombinant proteins on the *Bacillus subtilis* spore surface[J]. Protein Expression and Purification, 2014, 95(3): 67-76.

[23] Ricca E, Baccigalupi L, Cangiano G, et al. Mucosal vaccine delivery by non-recombinant spores of *Bacillus subtilis*[J]. Microbial Cell Factories, 2014, 13(1): 115.

[24] Donadio G, Lanzilli M, Sirec T, et al. Localization of a red fluorescence protein adsorbed on wild-type and mutant spores of *Bacillus subtilis*[J]. Microbial Cell Factories, 2016, 15(1): 153.

[25] Batista MT, Souza RD, Paccez JD, et al. Gut adhesive *Bacillus subtilis* spores as a platform for mucosal delivery of antigens[J]. Infection and Immunity, 2014, 82(4): 1414-1423.

[26] Mou C, Zhu L, Xing X, et al. Immune responses induced by recombinant *Bacillus subtilis* expressing the spike protein of transmissible gastroenteritis virus

in pigs[J]. Antiviral Research, 2016, 131: 74-84.

[27] Duc LH, Hong HA, Fairweather N, et al. Bacterial spores as vaccine vehicles[J]. Infection and Immunity, 2003, 71(5): 2810-2818.

[28] Liu Minggang, Dai Xixi, Xu Yuqin, et al. Study on the immune effect of recombinant *Bacillus subtilis* spores surface-displaying *Salmonella pullorum* OmpC using CotB as a molecular carrier in mice[J]. Chinese Journal of Preventive Veterinary Medicine, 2015, 37(12): 966-969.

[29] Song M, Hong HA, Huang JM, et al. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine[J]. Vaccine, 2012, 30(22): 3266-3277.

[30] Aps LRM, Diniz MO, Porchia BFMM, et al. *Bacillus subtilis* spores as adjuvants for DNA vaccines[J]. Vaccine, 2015, 33(20): 2328-2334.

[31] Aps LR, Tavares MB, Rozenfeld JH, et al. Bacterial spores as particulate carriers for gene gun delivery of plasmid DNA[J]. Journal of Biotechnology, 2016, 228: 58-66.

[32] Kim JH, Lee CS, Kim BG. Spore-displayed streptavidin: a live diagnostic tool in biotechnology[J]. Biochemical and Biophysical Research Communications, 2005, 331(1): 210-214.

[33] Richter A, Kim W, Kim JH, et al. Disulfide bonds of proteins displayed on spores of *Bacillus subtilis* can occur spontaneously[J]. Current Microbiology, 2015, 71(1): 156-161.

[34] Sirec T, Strazzulli A, Isticato R, et al. Adsorption of -galactosidase of *Alicyclobacillus acidocaldarius* on wild-type and mutant spores of *Bacillus subtilis*[J]. Microbial Cell Factories, 2012, 11(1): 100.

[35] Falahati-Pour SK, Lotfi AS, Ahmadian G, et al. Covalent immobilization of recombinant organophosphorus hydrolase on spores of *Bacillus subtilis*[J]. Journal of Applied Microbiology, 2015, 118(4): 976-988.

[36] Feng Fan. Surface display of human proinsulin on *Bacillus subtilis* spores and evaluation of its oral hypoglycemic efficacy[D]. PhD Dissertation. Zhenjiang: Jiangsu University, 2015, 64-78.

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