Application of *Bacillus subtilis* as a live vaccine vector: A review

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**ABSTRACT.** *Bacillus subtilis* is widely used as a probiotic in various fields as it regulates intestinal flora, improves animal growth performance, enhances body immunity, has short fermentation cycle, and is economic. With the rapid development of DNA recombination technology, *B. subtilis* has been used as a potential vaccine expression vector for the treatment and prevention of various diseases caused by bacteria, viruses, and parasites as it can effectively trigger an immune response in the body. In this review, we refer to previous literature and provide a comprehensive analysis and overview of the feasibility of using *B. subtilis* as a vaccine expression vector, with an aim to provide a valuable reference for the establishment of efficient vaccines.

**KEY WORDS:** *Bacillus subtilis*, expression vector, immunization, vaccine

**OVERVIEW OF BACILLUS SUBTILIS**

*Bacillus subtilis* is a gram-positive aerobic bacteria. It produces spores under harsh environmental conditions, which aid in the survival of its strains despite various environmental stresses. *Bacillus subtilis* as a probiotic regulates the intestinal flora of animals, improves their growth performance, and enhances host immunity.

Presently, the most commonly used host for the industrial production of heterologous proteins is *Escherichia coli* because it can be easily grown in large-scale fermentation, is genetically adaptable, and can produce large amounts of protein; however, the protein produced usually accumulates in the *E. coli* cells and forms inclusion bodies. Compared with *E. coli*, *B. subtilis* has a better fermentation capacity and produces numerous heterologous proteins. Moreover, *B. subtilis* without an outer membrane can secrete proteins directly into the culture medium, and thus, the secreted protein can be easily purified from the culture medium in its active form [37].

*Bacillus subtilis* can be used as a feed additive in breeding to improve animal production performance and provides economic benefits. Experiments have verified that the addition of 300 g/T *B. subtilis* to the piglet diet can improve the feed conversion rate and reduce the meat ratio by 5.3% compared with that of the control group [2], that is, the addition of 300 g/T *B. subtilis* in the diet group can provide an additional benefit of approximately $6 per pig [2]. A new study predicts that the global market for *B. subtilis* was $49 million in 2019 and is presumed to grow at a compound annual growth rate of approximately 8.5% in the next five years, thereby reaching $80 million by 2024 (https://www.eonmarketresearch.com/global-bacillus-subtilis-market-2019–33133).

With the rapid development of animal husbandry, aquatic products, and other industries in the past few years, the problem of antibiotic abuse has become more and more serious. Although antibiotics effectively treat bacterial diseases, their uncontrolled use can lead to antibiotic-resistant bacteria or “super bacteria”, thereby posing several challenges to the prevention and treatment of bacterial diseases. Probiotics are widely recognized for their pollution-free, residue-free, and growth-promoting characteristics. Several studies have reported probiotics as safe food supplements that can effectively replace antibiotics to prevent diseases and promote body growth. *Bacillus subtilis*, due to its ability to produce spores, can maintain a good number of viable bacteria even in extreme environments and endure long-term storage, and has thus become one of the major choices for use as a probiotic. Some scholars added *B. subtilis* to the daily diet of broilers to study the therapeutic effect of amikacin sulfate on *Salmonella* spp. The results revealed that adding $1.0 \times 10^8$ CFU/ml of *B. subtilis* to the diet can effectively replace amikacin sulfate with a cure rate of up to 88% in the experimental group [11].
BACILLUS SUBTILIS EXPRESSION SYSTEM

Since Spizizen established a method for preparing B. subtilis in a competent state in 1958, research on the expression system of B. subtilis has gained immense attention [23]. With the complete genome sequencing of 168 strains of B. subtilis, numerous foreign genes have been successfully expressed in the B. subtilis expression system [32].

As the research on the expression system of B. subtilis is relatively late compared to that of E. coli, some experimental techniques are not as efficient as those in the E. coli system. Bacillus subtilis as a probiotic has various advantages compared to E. coli as it is safe and nontoxic, can secrete foreign proteins, can survive in harsh environmental conditions to which E. coli cannot adapt, and can improve animal gut health (Table 1).

When B. subtilis is used as an expression host, the secretion of proteases is its major limiting factor since some proteases can degrade foreign proteins and reduce the yields of the B. subtilis expression system [12]. Some scholars deleted or mutated the corresponding protease genes to inactivate one or more proteases of the mutant, and found that the yield of foreign proteins by the mutant improved significantly with higher stability [20].

The stable vector skeleton ensures the efficient expression of foreign genes in B. subtilis. The content of endogenous genes in B. subtilis is limited, and the plasmid vectors used in the B. subtilis expression system are mainly from Streptococcus and Staphylococcus [3, 4, 13, 17, 20, 25, 28]. Based on their different replication methods, these plasmids can be classified into two types: butterfly replication type (θ replication type) and rolling circle replication type [9, 10, 16]. Although these two types of plasmids can replicate and express independently in B. subtilis, plasmid instability may occur [3], which limits its feasibility in practical applications. Presently, the third-generation plasmid is used, namely the E. coli–B. subtilis shuttle plasmid. For example, the shuttle plasmid Phb201 contains the plasmids Pta1060 and pUC19, which can be cloned in E. coli and then expressed in B. subtilis. The advantage of this type of carrier is that it is relatively stable in the continuous passage and fermenter culture [5]. A few shuttle plasmids, such as pHTo1 and pHTo43, induce the expression of foreign proteins secreted into the cell, whereas others, such as pHT43, induce the expression of foreign proteins secreted outside of the cell.

APPLICATION OF B. SUBTILIS AS A VACCINE CARRIER

As a vaccine carrier, B. subtilis can immunize animals through the nasal cavity, sublingual, oral and other routes, which can effectively induce immune response in these animals. Due to its excellent immunological properties, B. subtilis has gained immense attention as a vaccine carrier in preventing viral, bacterial, and parasitic diseases (Table 2).

ANTIVIRAL ASPECTS

During the past decade, the outbreaks of various viral diseases have severely affected the animal husbandry industry, causing huge economic losses to all countries, and have also seriously threatened human and animal life and health. Therefore, the research and development of various new vaccines has been crucial. Among the studies undertaken in this direction, several studies have reported that recombinant vaccines using B. subtilis as a carrier, for example, recombinant B. subtilis that can express the capsid protein (cap) of porcine circovirus, as established by Zhang et al. [34]. Recombinant B. subtilis that expresses the spike protein of transmissible gastroenteritis virus (TGEV) was established by Mou et al. [18] in 2016. Mou et al. [19] used the B. subtilis expression system to express the HA protein of the highly pathogenic avian influenza H5N1 virus. Recombinant B. subtilis spores capable of expressing the M2 protein of the influenza A virus were established by Tomasz et al. [14, 35]. In 2019, Wang et al. [29] employed recombinant B. subtilis to express the collagenase equivalent domain protein (COE) of porcine epidemic diarrhea virus (PEDV). In 2019, Wang et al. [30] established a recombinant B. subtilis, which can express the glycoproteins (GC and GD) of the pseudorabies virus (PRV). Moreover, in 2019, Aghaei et al. [1] used B. subtilis as a vector to express the foot-and-mouth disease virus (FMDV) proteins, VP1 and 3A.

Bacillus subtilis has several applications as a vaccine carrier for porcine viral diseases. Porcine circovirus type 2 (PCV2) is the causative agent of postweaning multiple system failure syndrome and is associated with various diseases. PCV2 is widely distributed in the pig industry, causing serious economic losses. Studies have reported that the PCV2 cap and the protein expressed by the major antigen gene ORF2 are all immunogenic and can be potentially used for vaccine development [34]. Zhang et al. successfully constructed a recombinant B. subtilis strain that can express PCV2 cap, and further evaluated the immune effects of

Table 1. Comparison of the characteristics of Bacillus subtilis and Escherichia coli expression systems

| Features              | Bacillus subtilis | Escherichia coli |
|-----------------------|-------------------|-----------------|
| Safety                | Nontoxic and safe | Partly toxic    |
| Vitality              | Can form spores, strong survivability | Weak ability to survive under harsh conditions |
| Secretion ability     | Secrete proteins directly into the culture medium | Easy to form inclusion bodies |
| Probiotics            | Probiotic microorganisms | Nonprobiotic |
| Research history      | Shorter than E. coli | Very long |
the recombinant bacteria on newborn piglets via oral immunization [34]. The results revealed that after the oral administration of recombinant bacteria, PCV2-specific IgA levels in piglet digestive tract and IgG levels in serum increased. Meanwhile, the expression of TLR2, TLR9, interleukin 1β (IL-1β), and interleukin 6 (IL-6) also increased. The secretion of both interferon-γ and β-defensin 2 increased. Moreover, the recombinant bacteria stimulated bone marrow-derived dendritic cell maturation and T cell proliferation.

PEDV is the causative agent of porcine epidemic diarrhea, which is characterized by highly fatal acute diarrhea in piglets, thereby resulting in huge losses to the pig industry worldwide. The pathogen PEDV belongs to the family of porcine coronaviruses (CoVs). PEDV is mainly transmitted via the digestive tract and destroys the host intestinal mucosal surface by infecting the intestinal epithelial cells. Wang et al. constructed a recombinant B. subtilis that could express PEDV COE and evaluated its immunogenicity in piglets [29]. The results indicated that compared with the control group, the cytokines IL-1β and IL-10 were markedly upregulated in piglets immunized orally with recombinant B. subtilis, and the proportion of CD4+/CD8+ T-cell ratio was also remarkably enhanced.

TGEV is the causative agent of transmissible gastroenteritis that causes severe diarrhea in suckling pigs, thereby leading to huge economic losses to the pig industries worldwide. Mou et al. constructed a recombinant B. subtilis that can express the spike protein of TGE and orally immunized piglets to evaluate their immunogenicity [18]. The results revealed that the recombinant bacteria can effectively increase the specific SIgA titer in piglet feces, and IgG and neutralizing antibody titers in serum. Moreover, it can recruit more dendritic cells to induce an immune response.

Pseudorabies caused by PRV infection severely hampers the biological safety of both animals and humans. Wang et al. successfully constructed recombinant B. subtilis expressing the dominant antigen regions of PRV gC and gD proteins (named B. subtilis-gCa and B. subtilis-gDa) and evaluated the immunogenicity of the two recombinant bacteria in mice using a nasal drip [30]. The results revealed that B. subtilis-gCa and B. subtilis-gDa can effectively stimulate the immune response of IgG and IgA, and trigger specific T lymphocyte proliferation by regulating interferon-γ (IFN-γ) and IL-10, and may eventually produce a high titer of neutralizing antibodies against the PRV infection in mice. Moreover, compared with B. subtilis-gCa, B. subtilis-gDa has a stronger immune effect.

Bacillus subtilis further shows promising applications as a vaccine carrier for avian viral diseases. The HPAI virus H5N1 is a global threat to the poultry industry. The virus has evolved rapidly, presenting a high degree of genetic diversity and a wide range of hosts; it rapidly spreads among birds, with severely effects for the poultry industry. Mou et al. constructed a recombinant B. subtilis that can express the H5N1 hemagglutinin (HA) protein (BS-HA) [19], and evaluated its immune effect in chickens via oral immunization. The results revealed that the oral immunization of BS-HA in chickens remarkably increases the weight of small intestinal villi, height of small intestine, and area of lymphoid tissue in ileum. Moreover, BS-HA induces tracheal and intestinal cytokine secretion and Toll-like receptor expression. Furthermore, BS-HA increases specific IgA antibody titers in chicken trachea and IgG and HI antibody titers in serum. Song et al. reported that when the killed spores of B. subtilis were combined with virions and administered intranasally to mice [22], the levels of systemic IgG and mucosal IgA increased, with the highest increase in the level of IgG2a (Th1 antibody type). In this experiment, mice dosed twice with 20 ng of HA (hemagglutinin) inactivated spores were completely protected against 20 LD50 of H5N2 virus.

B. subtilis has applications as a vaccine carrier for other viral diseases as well. Influenza viruses belong to the Orthomyxoviridae family. Du et al. successfully constructed a recombinant spore that can anchor the M2e influenza virus antigen on the

| Table 2. Application of Bacillus subtilis as a vaccine carrier |
|-------------------------------------------------------------|
| **Target pathogen** | **Antigen protein** | **Immune route** | **References** |
| Virus | | | |
| PCV2 | Capsid protein | Oral | [34] |
| PEDV | Collagenase equivalent domain protein | | [29] |
| TGEV | Spike protein | | [18] |
| PRV | gC protein, gD protein | | [30] |
| HPAIV | HA protein | | [19] |
| Influenza A virus | M2 protein | | [14, 35] |
| FMDV | VP1 protein, 3A protein | | [1] |
| Bacteria | Streptococcus agalactiae | Sip protein | Oral | [33] |
| Clostridium difficile | FliD protein | Oral | [21] |
| Salmonella | OmpC | Oral | [6] |
| Helicobacter pylori | UreB protein | Oral | [36] |
| Mycoplasma tuberculosis | Truncated protein 21 | Nasal | [7] |
| Mycoplasma hypopneumoniae | P97R1, P46 protein | Intranasal | [31] |
| Parasitic | Clonorchis sinensis | CsPmy protein | Intraperitoneal, Intragastric | [24] |
| Echinococcus granulosus | Cysteine protease | Oral | [26] |
| | EgTrp, EgA31 protein | Oral | [27] |

Existing recombinant B. subtilis and its expressed antigen.
surface of the bun shell by fusing the capsid protein (CotB, CotC, CotZ, and CgeA). Among these [14], CotC and CotZ had the highest efficiency and CotB, CotZ, and CgeA recombinant spores could induce mouse serum producing specific antibodies; however, none of the four recombinant spores can induce the production of specific secreted IgA antibodies (SlgA) in the lung tissue and trachea of mice. Despite this, the system is still attractive and should be appropriately improved to enhance its immunogenicity. Zhao et al. reported that after oral administration of recombinant spores expressing M2 protein [35], the IgG and slgA antibody titers of mice were markedly increased, thereby indicating that the recombinant spores have the ability to induce protective immunity. Foot-and-mouth disease (FMD) is a highly contagious livestock disease. It is important to control the disease by vaccination against O, A, and Asia 1 serotypes. VP1 (structural) protein and 3A (nonstructural) protein are crucial in FMDV infection and can be used to design recombinant vaccines. Aghaei et al. constructed codon optimized VP1 [141–160] -GS-VP1 [23–42] -GS-3A [21–35] -GS, and cloned it into the pHT43 shuttle vector, which was further expressed in B. subtilis strain WB600 [1]. The results revealed that the recombinant protein has epitope characteristics and can be used as a candidate vaccine to control all serotypes of FMD in Iran.

**ANTIBACTERIAL EFFECTS**

*Bacillus subtilis* isolated from the rhizosphere of plants has been used to control plant diseases for decades and can induce resistance to plant pathogens [8]. *Bacillus subtilis* is widespread in nature, does not cause disease or pollutes the environment, and can be used as a probiotic to resist bacterial contamination and prevent bacteria from developing drug resistance. *Bacillus subtilis* has been used as a vaccine carrier to prevent bacterial diseases, for example, recombinant *B. subtilis* spores capable of expressing *Streptococcus agalactiae* sip protein were established by Yao et al. [33]. Recombinant spores, which were capable of presenting *Clostridium difficile* antigen (FilD protein), and adjuvant (human IL-1β fragment VQGEESNDK peptide) were simultaneously established by Potocki et al. [21]. Recombinant *B. subtilis* expressing the OmpC protein of *Salmonella* was established by Dai et al. [6]. Recombinant *B. subtilis* spores capable of expressing UreB protein of *Helicobacter pylori* were established by Zhou et al. [36]. Recombinant *B. subtilis* spores capable of expressing *Mycobacterium tuberculosis* MT truncated protein 21 fusion protein were established by Das et al. [7], and recombinant *B. subtilis* capable of expressing P97R1 and P46 antigen proteins of *Mycoiplasma pneumoniae* was established by Wang et al. [31].

The applications of *B. subtilis* in protection against other gram-positive bacteria are presented here. Due to the obvious morbidity and mortality, *S. agalactiae* infection is posing serious issues in aquaculture; this limits the healthy development of *S. agalactiae* morbidity and mortality, has been used as a vaccine carrier to prevent bacterial diseases, for example, recombinant *B. subtilis* spores capable of expressing *Streptococcus agalactiae* sip protein were established by Yao et al. [33]. Recombinant spores, which were capable of presenting *Clostridium difficile* antigen (FilD protein), and adjuvant (human IL-1β fragment VQGEESNDK peptide) were simultaneously established by Potocki et al. [21]. Recombinant *B. subtilis* expressing the OmpC protein of *Salmonella* was established by Dai et al. [6]. Recombinant *B. subtilis* spores capable of expressing UreB protein of *Helicobacter pylori* were established by Zhou et al. [36]. Recombinant *B. subtilis* spores capable of expressing *Mycobacterium tuberculosis* MT truncated protein 21 fusion protein were established by Das et al. [7], and recombinant *B. subtilis* capable of expressing P97R1 and P46 antigen proteins of *Mycoplasma pneumoniae* was established by Wang et al. [31].

The results revealed that the spleens of mice immunized with the recombinant antigen/adjuvant spores promote the production of cytokines IL-2, IL-4, IL-6, IL-17A, tumor necrosis factor-α (TNF-α), and IFN-γ, indicating that these spores can effectively elicit immune response.

Tuberculosis is an important cause of morbidity and mortality worldwide. Tuberculosis caused by the invasion of *Mycobacterium tuberculosis* is a highly contagious chronic disease that has adverse effects on the health of humans and animals. Das et al. constructed two recombinant spores expressing *M. tuberculosis* truncated protein 21 fusion protein on the surface of spores or in the cytoplasm of *B. subtilis* [7]. After nasal immunization, both spores delivered antigen to mouse dendritic cells, and relatively high levels of Ag85B-specific IgG antibodies in mouse serum were detected. These results indicate that *B. subtilis* spores are ideal carriers for antigen delivery and have immense potential in the development of primary antituberculosis and booster vaccines.

*Bacillus subtilis* also has applications in protection against gram-negative bacteria. Salmonellosis is a major public health problem worldwide. Therefore, various control strategies for Salmonella infection are urgently warranted. The outer membrane protein (Omp) of Salmonella is one of the important components of Salmonella antigens. It has strong immunogenicity and can trigger an immune response in the body. Studies have reported that the seven outer membrane proteins of Salmonella OmpC, PagC, OmpX, NmpC, FadL, TolC, and BtuB provide good immune protection. Dai et al. immunized mice with recombinant B. subtilis spores expressing the outer membrane protein OmpC of Salmonella [6], and evaluated the immune effect of the recombinant spores on the mice. The results revealed that the immunization of mice with recombinant spores can stimulate significant anti-OmpC serum IgG and intestinal mucosal SlgA responses, and that the level of the effective protection against lethal attacks remains high. *Helicobacter pylori* is a pathogenic spiral bacterium that causes chronic infections in humans, with a high infection rate. *H. pylori* infection is closely related to certain diseases, such as chronic gastritis, peptic ulcer, gastric adenocarcinoma, and lymphoid tissue lymphoma associated with mucosa. Zhou et al. established recombinant *B. subtilis* spores expressing the *H. pylori* protein UreB, and evaluated their immunogenicity by orally immunizing mice [36]. The results revealed that the oral administration of the recombinant spores induced an increase in the levels of UreB-specific IgG in serum and UreB-specific IgA in feces, and increased the levels of IL-10 and IFN-γ in splenocytes. Moreover, these spores can remarkably reduce gastric *H. pylori* bacterial load (80.0%). These results indicate that recombinant spores can improve the health of mice infected with *H. pylori*. 
**APPLICATION OF BACILLUS SUBTILIS**

*Mycoplasma hyopneumoniae* is the causative agent of swine band pneumonia, a chronic respiratory disease that affects pigs of all ages. Wang et al. constructed a recombinant *B. subtilis* (BS-P97R1, BS-P46) [31], which can express the P97R1 or P46 antigen of *M. hyopneumoniae* and evaluated its immune effect on BALB/c mice. The results indicated that the immunoglobulin bronchoalveolar lavage fluid (BAL) induced strong P97R1-specific and P46-specific immunoglobulin G (IgG) and secreted immunoglobulin A (SIgA) antibodies. Furthermore, the levels of specific IL-4 and IFN-γ increased in immunized mice, and the proliferation of lymphocytes also increased.

### ANTIPARASITIC ASPECTS

Due to the unique biological characteristics of parasites, it is difficult to develop antiparasitic vaccines. Vaccination against parasitic diseases is still in its infancy, and various issues need to be resolved; however, some reports reveal the use of *B. subtilis* as a vaccine carrier for protection against various parasitic infections, for example, recombinant *B. subtilis* spores established by Sun et al. [24] that can express Clonorchis sinensis paramyosin (CsPmy), those constructed by Tang et al. that can express *C. sinensis* cysteine protease [26], and recombinant *B. subtilis* spores that can secrete promyosin [EgTrp] and paramyosin [EgA31] of Echinococcus granulosus as constructed by Vogt et al. [27].

Clonorchis sinensis is an important fish-borne zoonotic parasite that threatens public health and has important socioeconomic significance in endemic areas. Over the years, clonorchiasis caused by *C. sinensis* has posed several health issues. Sun et al. [24] established recombinant *B. subtilis* spores capable of expressing CsPmy, and studied the immune response of the recombinant spores in a mouse model via intraperitoneal injection and intragastric administration. The results revealed that the level of specific IgG antibodies in serum and intestinal mucosa increased, indicating the induction of a systemic immune response. Compared with the intraperitoneal injection immunization group, the IgG, intestinal mucus, feces, and bile slgA content in the intestinal mucus of gastric immunized mice were higher. Tang et al. [26] constructed recombinant *B. subtilis* spores capable of the surface expression of *C. sinensis* cysteine protease, and tested the immunogenicity of the recombinant spores in mice via subcutaneous and oral immunization. The results revealed that the level of specific IgG1 in the serum of subcutaneously immunized mice markedly increased, and after 2 and 4 weeks, the level of IgG2a was remarkably increased. Oral immunization could also induce both local and systemic immune response in the mice.

Echinococcus granulosus, causative agent of echinococcosis, belongs to the genus *Echinococcus*. The adults are parasites of canine carnivores, while the larvae (echinococcus) are parasites of humans and various herbivorous livestock and other animals, and cause a serious zoonosis, called echinococcosis or hydatidosis. Vogt et al. [27] constructed recombinant *B. subtilis* spores that can secrete the proteins promyosin (EgTrp) and paramyosin (EgA31), and evaluated their immunogenicity in dogs. The results revealed that the oral recombinant *B. subtilis* spores can cause specific humoral reactions in dogs.

### IMMUNITY ENHANCEMENT

In recent years, it has been found that both *B. subtilis* cells and spores have the potential to be used as immune adjuvants and can be used in conjunction with other immune adjuvants to effectively improve immune response. It has been reported that the glycosylated antibacterial peptide sublancin produced by *B. subtilis* possesses antibacterial and immunomodulatory activity. Liu et al. [15] studied the effect of sublancin on the immune function and serum antibody titer in specific pathogen-free broilers vaccinated with Newcastle disease vaccine. The results indicated that sublancin can promote B lymphocyte proliferation and increase CD8+ T lymphocyte levels. Moreover, sublancin has the potential to induce IFN-γ, IL-10, and IL-4 secretion.

### OUTLOOK

Presently, with the advancements in biomedical technology, the construction of *B. subtilis* vectors has improved; however, with the further development of genetic engineering, *B. subtilis* expression vectors will find newer and greater applications.

1. Smaller, higher copy number vector plasmids. The size of the plasmid affects the cloning operation, and the copy number determines the amount of expression.
2. Vector plasmid with higher stability. Stable plasmid vectors form the basis for foreign genes to be expressed.
3. Further improvement of operational methods. Compared to *E. coli*, the operation of *B. subtilis* needs improvement, specifically the experimental operation method.
4. The establishment of an expression system with simpler and more effective control methods. For example, temperature, pressure, and other adjustments are adopted, the method is simple, and the cost is low.
5. Establishment of efficient secretion system. On the basis of good expression vectors, the adaptability of signal peptides to foreign genes needs to be systematically studied in order to find the most suitable signal peptides, and achieve efficient secretion and expression of foreign genes.

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