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Display of *Bombyx mori* Nucleopolyhedrovirus GP64 on the *Bacillus subtilis* Spore Coat

Guohui Li · Qi Tang · Huiqing Chen · Qin Yao · Degang Ning · Keping Chen

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Abstract To investigate whether *Bombyx mori* immunized with *Bacillus subtilis* spore displaying GP64 escape from the *B. mori* nucleopolyhedrovirus (*Bm*NPV) attack, a recombinant integrative plasmid named pJS700-*GP64* was constructed, which carries a recombinant *cotC-Gp64* gene under the control of the *cotC* promoter. In this study, pJS700-*GP64* was transformed into *B. subtilis* 168 (trp^-) competent cells, an amylase (*amyE*) inactivated mutant was selected, and was confirmed to be a double cross-over integrant, *cotC-Gp64* fragment of which was integrated into *B. subtilis* chromosome. Gp64 was expressed on the spore surface and recognized by Gp64-specific antibody. Results of *B. mori* when challenged with *Bm*NPV indicated that *B. mori* vaccinated with the recombinant spores possessed resistance to the invasion of *Bm*NPV at some degree.

Introduction

The silkworm, *Bombyx mori* is an important economic insect and lepidopteran model insect. At present, over 30 million farmer households are involved in sericultural production in China across ten provinces. Unfortunately, the silkworm, *B. mori* is particularly susceptible to virus diseases, especially due to *B. mori* nucleopolyhedrovirus (*Bm*NPV), which results in great loss in sericulture [1]. *Bm*NPV is a member of the Baculoviridae family, which has an enveloped, circular, and double-stranded DNA ranging from approximately 80–180 kbp. They infect only invertebrates, and the majority of baculoviruses described are from insects in the order Lepidoptera [2].

The envelope glycoprotein, GP64, of *Bm*NPV is a type I integral membrane protein that is present on the infected cell surface and on the virion. It is a highly conserved gene among the group I *alphabaculoviruses* [3, 4]. GP64 was also a viral fusion protein mediating pH-triggered membrane fusion during virus entry by endocytosis. Evidences suggested that GP64 was necessary and sufficient for pH-dependent membrane fusion during viral entry. In addition, GP64 is also necessary for efficient budding and production of infectious virions [5, 6]. Recently, a region important for receptor-binding was mapped onto the N-terminal portion of the GP64 ectodomain [7].

Bacillus subtilis is a Gram-positive bacterium, which has been widely used as a model organism for laboratory studies, especially of sporulation. In addition, this organism is not pathogenic for both human and animal, and has been adopted as a probiotic added in the consumption. Previous findings indicated that *B. subtilis* spore was a powerful vehicle for delivery of heterologous antigens or some bioactive molecule [8, 9]. In this article, an integrative *B. subtilis* strain, which exhibited GP64 on the *B. subtilis* spore surface, has been successfully constructed, and the antiviral effect of *B. mori* orally immunized with *B. subtilis* spores displaying GP64 examined.

Guohui Li and Qi Tang have contributed equally to this study.

G. Li · Q. Tang · H. Chen · Q. Yao · K. Chen (✉)
Institute of Life Sciences, Jiangsu University, 301# Xuefu Road, Zhenjiang 212013, China
e-mail: kpchen@ujs.edu.cn

D. Ning
School of Environment, Jiangsu University, 301# Xuefu Road, Zhenjiang 212013, China

Materials and Methods

Bacterial Strains and Transformation

B. subtilis strain 168 (trp$^-$) was obtained from Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University. Preparation and transformation of *B. subtilis* strain 168 (trp$^-$) competent cells was performed as previously described [10]. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *E. coli* competent cells were carried out in the *E. coli* strain DH5α. Expression of target protein GP64 was carried out in *E. coli* strain DE3 cells. Bacterial strains were transformed as previously described [11].

Expression of GP64 in *E. coli* and Preparation of GP64-Specific Antibody

Two primers *GP64*-F1: 5′-ATGAATTCAATCAGTCATACCAAGGCTTCGA-3′ (*Eco*RI site was underlined) and *GP64*-R1: 5′-GCAAGCTTCCAAGTGGGTGGCCG -3′ (*Hin*dIII site was underlined) were designed to amplify a 904-bp fragment of *Bm*NPV GP64. The PCR product was cloned first into pMD18-T (TaKaRa) and then into the expression vector pET28a (Promega), which generated plasmid pET28a-*GP64* with 6×*His*-tag sequence at the N-terminus. *E. coli* DE3 cells containing pET28a-*GP64* were grown to an optical density at about 0.6 of OD_{600} and induced by addition of 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG). After incubation for 10 h at 37°C, cells were harvested by centrifugation at 7,000 g for 15 min at 4°C. The fusion protein present in the pellet was separated in 15% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. The induced GP64 band was excised directly, and the antiserum was raised in rats according to the method of Sambrook et al. using Freund's adjuvant [11].

Construction of Plasmid pJS700-GP64 and Site-Specific Gene Integration in *B. subtilis amyE* Locus

To obtain an integration of fusion gene *cotC-GP64* in *B. subtilis amyE* locus, a recombinant plasmid for double cross-over with *B. subtilis* chromosome was constructed. The authors first amplify a 1489-bp fragment from *Bm*NPV genomic DNA with primer pair *GP64*-F2: 5′-CCGGTACCTGCAACGCGCAAATGA-3′ (*Kpn*I site was underlined) and GP64-R2: 5′-CGGAATTCTTAATATTGTCTACTATTACGGTTTC-3′ (*Eco*RI site was underlined). The PCR product was digested with *Kpn*I and *Eco*RI and then was cloned into vector pJS700 (Ning et al. published) to generate the recombinant plasmid named pJS700-*GP64*, in which the upstream or downstream flanking region of Erythromycin (Em)-*cotC-GP64* was homologous to *B. subtilis amyE*, and it was subsequently verified by restriction analysis and complete sequencing.

Plasmids pJS700-*GP64* were digested with enzyme *Bgl*II and *Bam*HI, and the resulting linear fragment containing Em-*cotC-GP64* gene cassette and *amyE* flanking region was gel purified, resuspended in distilled water to a final concentration of 200 ng/μl. 20 ng of target fragment was used to transform the competent cells of *B. subtilis* strain 168 (trp$^-$). The transformed cells were incubated at 37°C, 80 rpm for 30 min in 1 ml LB medium, then 100 μl was spread onto LB medium containing Em 0.4 μg/ml. Plates were incubated at 37°C overnight, and colonies resistant to Em were selected, and target colony with *cotC-GP64* integrated in *B. subtilis amyE locus* was analyzed by amylase activity and PCR confirmation.

Erythromycin-resistant (Em^r) clones were the result of a double cross-over recombination, resulting in the interruption of the non-essential *amyE* in the *B. subtilis* chromosome (Fig. 2a). Several Em^r colonies were selected and were grown on LB plates containing 1% starch overnight, and then the plates were stained with iodine to examine the amylase activity. A blue color was produced by starch in the presence of free iodine when fusion gene *cotC-GP64* was integrated in the *B. subtilis* chromosome. However, no blue color was observed when iodine was added in the starch plates, owing to the expression of *amyE* from *B. subtilis* 168 (trp$^-$) chromosome hydrolyzing the starch in the plates.

Interruption of *amyE* from its locus in *B. subtilis* chromosome and correct insertion of *GP64 and cotC partner* at the *amyE* locus were confirmed by PCR. The relative positions of the primer pairs are shown in Fig. 2b. Primers *amyE-F* and *amyE-R* will amplify a 1098-bp wild-type fragment and an 4.5-kb integrated fragment. Primer pair *GP64*-F2 and *GP64*-R2 were used to detect the correct insertion of the *GP64* gene. Primer pairs *amyE*-F/*GP64*-R2 and *GP64*-F2/*amyE*-R were used to examine the junction between the upstream or downstream flanking region and *GP64*.

Preparation of *B. subtilis* Spores

Sporulation of wild type *B. subtilis* 168 (trp$^-$) and recombinant strain DRJS711 (*cotC-GP64*) was induced in DSM (Difco-sporulation media) using the exhaustion method as previously described [12]. Cultures were harvested 48 h after the initiation of sporulation. Spores were collected, washed several times, and purified by lysosome treatment as described by Nicholson and Setlow [12] to break any residual sporangial cells. PMSF was added to inhibit proteolysis. After the final suspension of spores in water treated at 68°C for 1 h to kill any residual cells, the

number of purified spores harvested was measured by direct counting using hemocytometer under an optical microscope.

Immunodetection of GP64 in the Extraction from *B. subtilis* Spore Coat Proteins

Spore coat proteins were extracted from spores suspensions of strain DRJS711 or 168 (trp^-) at high density (1×10^{10} spores/ml), using an SDS–DTT extraction buffer as previously described [12]. Extracted proteins were assessed for concentration using the Bio-Rad DC Protein Assay kit.

To confirm that GP64 was expressed on the *B. subtilis* spore surface of strain DRJS711, culture was harvested after 48 h of the initiation of sporulation. Then, the spore coat proteins were extracted from strain DRJS711 or 168 (trp^-), mixed with 1× SDS-PAGE loading buffer and analyzed by Western blot. Gp64 antibodies and pre-serum were used at a dilution of 1:1000. Immunoreactive proteins were visualized using goat anti-rat IgG and horseradish peroxidase. Antibodies against the GP64 protein were used to perform western blot analysis. Western blot filters were visualised by the enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instruction.

Larval Bioassays

In order to examine whether *B. subtilis* spore displaying GP64 has any effect on inhibiting the *Bm*NPV attack in *B. mori*, bioassays were performed as described following. In brief, spores were harvested from DSM culture, purified by centrifugation, and resuspended in double-distilled water. To determine the antiviral effect of recombinant spores, 300 µl samples containing 0 (control), 1×10^3, 1×10^4, and 1×10^5 *B. subtilis* spores separately, were applied onto three mulberry leaves. Newly molted second-instar larvae of *B. mori* were reared at 25°C in canteen and were fed on leaves sprayed with above concentrations of 168 (trp^-) wt or DRJS711 recombinant spores. After 12 h, fresh mulberry leaves contaminated with 1×10^6 *Bm*NPV PIBs were added into the canteens when the remaining diet sprayed with spores was cleared out. Fresh diet was added into the canteens when all the virus-contaminated diet was consumed. After 72 h, the number of *B. mori* with typical symptom of *Bm*NPV infection was recorded. Thirty larvae per dosage were used in the experiment, and each dose was repeated in triplicate. Statistic analysis was performed using the Student's *t* test. *P* value of <0.05 was considered statistically significant.

Results

Construction of Recombinant Plasmid pJS700-GP64

A 1489-bp fragment was amplified from *Bm*NPV genomic DNA by specific primer pair *GP64*-F2 and *GP64*-R2 (Fig. 1b Lane 1). The PCR products obtained were cloned into pJS700 to generate the recombinant plasmid pJS700-*GP64* (Fig. 1a), in which *GP64* and *cotC* are co-expressed under the control of the *cotC* promoter. The

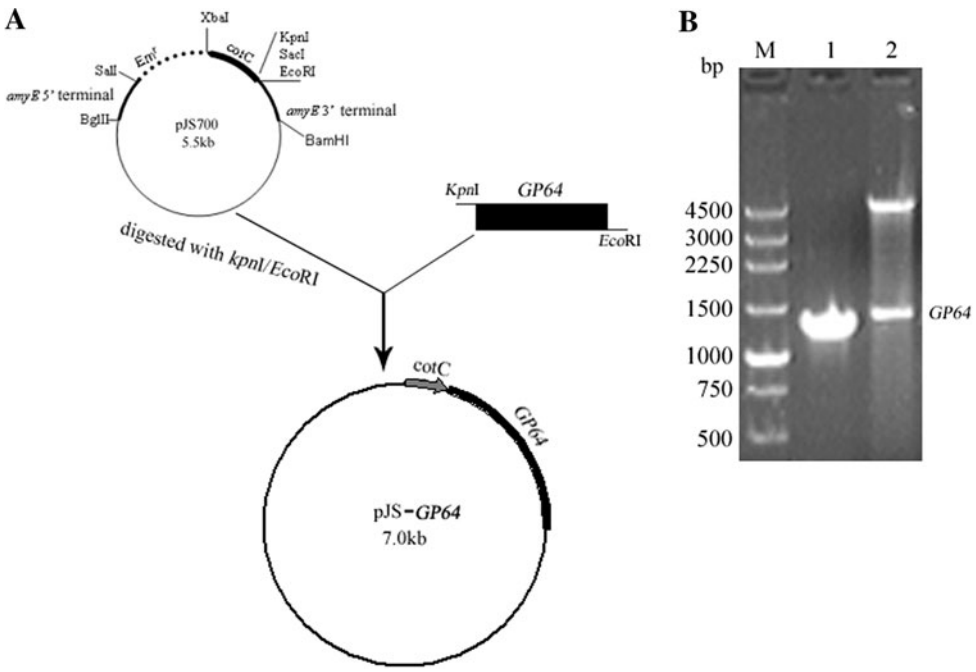

Fig. 1 Schematic diagram showing the construction of integrative recombinant plasmid pJS700-*GP64* and identification of pJS700-*GP64* by PCR and enzyme digestion. **a** The flow chart of plasmid pJS700-*GP64* construction. *amy*E 5′-terminal and *amy*E 3′-terminal are integrative fragments from amylase gene of *B. subtilis* 168 (trp^-), Em^r gene resistant to erythromycin; *cotC B. subtilis* spore coat. **b** *Lane M* DNA marker; *Lane 1* 1489-bp fragment product amplified from *Bm*NPV genomic DNA; *Lane 2* restriction analysis of pJS700-*GP64* with *Kpn*I and *Eco*RI

resulting *pJS700-GP64* was subjected to the analysis of restriction–digestion (Fig. 1b Lane 2) and subsequent sequencing. The size of obtained sequences matched with the expected fragments of *Bm*NPV *GP64*.

Identification of GP64 Integrated in *B. subtilis* DRJS711 Chromosome

To exhibit GP64 on the spores surface of *B. subtilis*, a recombinant *B. subtilis* DRJS711 was generated, in which fusion gene *cotC-GP64* was integrated into the *B. subtilis* chromosome at the *amy*E locus by double cross-over recombination events (Fig. 2a).

Positive colonies with *cotC-GP64 integrated in B. subtilis amyE locus* were screened primarily by analysis of amylase activity. Several *B. subtilis* colonies with integrative chromosome were identified by blue color, but control with no integration in *B. subtilis* chromosome were identified by no blue color (Fig. 2c).

The disruption of *amyE* was further confirmed by PCR with several primer pairs (Fig. 2d). Primer pair *amy*E-F/*amy*E-R produced a 4.5 kb product from the DRJS711 chromosome, in comparison with a 1098-bp product from the 168 (trp$^-$) chromosome. Primer pair *GP64*-F2/*GP64*-R2 produced 1489-bp product in DRJS711 chromosome, but no PCR product in 168 (trp$^-$) chromosome. Primer pair *amyE*-F/*GP64*-R2 produced 3.8 kb product in DRJS711 chromosome, but no PCR product in 168 (trp$^-$) chromosome. Primer pair *GP64*-F2/*amyE*-R produced 2.3 kb product in DRJS711 chromosome, but no PCR product in 168 (trp$^-$) chromosome.

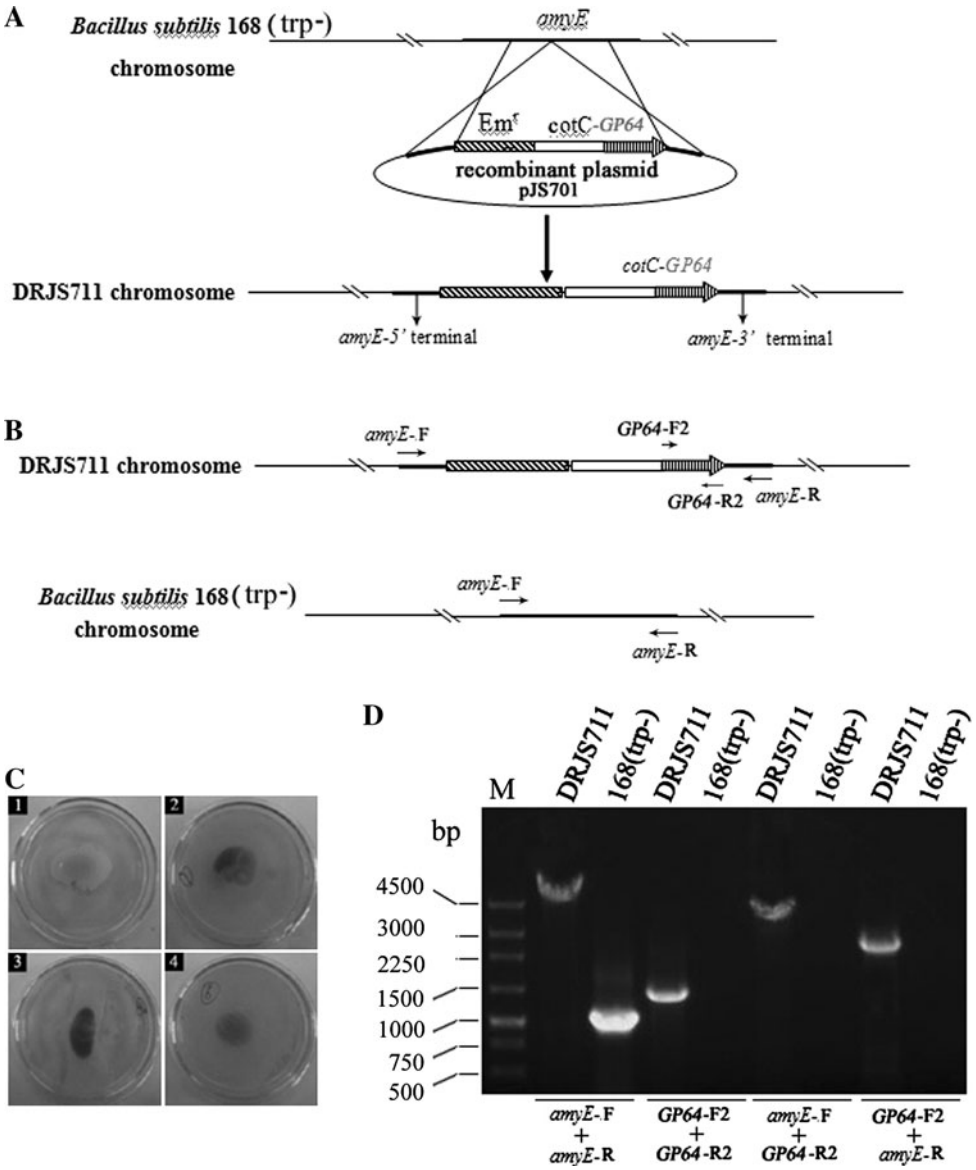

Fig. 2 Strategy for construction of fusion gene *cotC-GP64* integrated in *B. subtilis amyE* locus and by analysis of amylase activity and PCR confirmation. **a** Schematic diagram showing the structure of *amy*E locus in wild type and integrative chromosome and the integration of fusion gene *cotC-GP64*. **b** Positions of primer pairs used in the confirmation of the *GP64* gene insertion. **c** Identification of recombinant strains by analysis of amylase activity. *1 B.subtilis* 168 (trp$^-$) as a control; *2*, *3*, and *4* recombinant strain with *amy*E disruption. **d** PCR analysis of different primer pairs. *M* DNA marker with sizes indicated. The virus templates are shown *above* each *lane*, and the primer pairs used are shown *below*

Surface Display of GP64 on the Recombinant Spores

To confirm that GP64 is expressed on the spore surface, antibodies against the GP64 protein were used to perform western blot analysis. The result showed that a 69 kDa band was detected in the extracts from recombinant spores, while no similar band in the extracts from wild-type spores was detected, indicating the presence of the cotC–GP64 fusion protein in the spore coat (Fig. 3).

B. mori Larvae Following Oral Administration of Recombinant Spores Challenged with BmNPV Attack

To test for induction of local and systemic immunity, newly molted second-instard *B. mori* larvae were immunized orally with either a suspension of wild type *B. subtilis* 168 (trp^-) spores or recombinant spores of strain DRJS711 respectively. The *B. mori* defense against *Bm*NPV attack results showed that there was significant difference ($P < 0.05$) between recombinant spores group (percentage of infection, 53.3%) and wild spores or control group (percentage of infection, 73.3%) (Fig. 4).

Discussion

B. subtilis display system is based on the construction of heterologous DNA fused to spore coat gene such as *cotB*, *cotC*, and *cotG* expressed on the surface of spores, which has been proven to be a novel and potentially powerful system to display heterologous antigens. Heterologous proteins and peptides being displayed on the surface of *B. subtilis* is becoming increasingly important, which has been used as a tool for fundamental and applied research in microbiology, molecular biology, vaccinology, and biotechnology.

Previous findings indicated that many antigens had been successfully exhibited on the surface of *B. subtilis* spores, including tetanus toxin fragment C (TTFC) of *Clostridium*

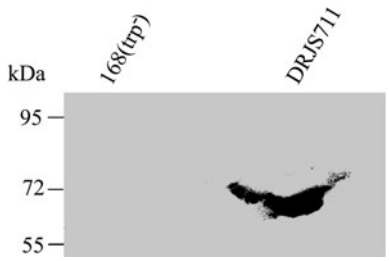

Fig. 3 Western blot analysis of proteins extracted from purified spores of strains 168 (trp^-) or DRJS711. Extracted spores coat used for Western blot are indicated *above* the *lanes*. The protein standards are indicated on the *left*

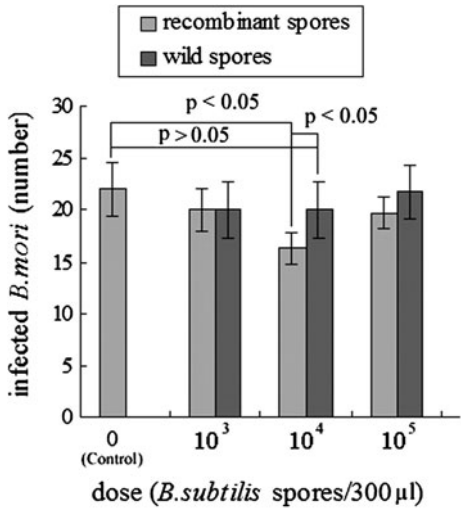

Fig. 4 Groups of *B. mori* were challenged with *Bm*NPV after oral vaccination with *B. subtilis* spores. Dose of *B. subtilis* spores were indicated *below*. Data were presented as arithmetic means ± standard deviations. Each sample was performed in triplicate

tetani, the B subunit of the heat-labile toxin of *E. coli* (LTB) and alpha toxin of *Clostridium perfringens* [13–16]. In this work, *GP64* from *Bm*NPV was fused to the *cotC*, a major component of the *B. subtilis* spore coat, as a fusion partner for the expression of heterologous protein, and integrated into the *B. subtilis* chromosome at the *amy*E locus by double cross-over recombination. Western blot analysis against extracted spore coat proteins indicated that GP64 was successfully displayed on the *B. subtilis* spores.

Although it is arguable as to whether acquired immunity exists in invertebrates, some evidences indicated that invertebrates have a true adaptive immune system. For example, the shrimp vaccinated with VP 26 or VP 28 possessed resistance to White Spot Syndrome and reduced significantly the cumulative mortality of shrimp [17, 18]. Challenging successive mosquito generations with a densonucleosis virus yields progressive survival improvement, indicating that a specific, adaptive manner may be employed to reduce the incidence and severity of disease in arthropods [19].

Heterologous proteins and peptides displayed on the surface of *B. subtilis* spores was used for oral dosing and shown to generate specific systemic and mucosal immune responses. Based on the above opinion, we consider that *B. mori* may also employ a currently unknown specific adaptive manner in vivo. Therefore, GP64 was designed to display on the surface of *B. subtilis* spores using *cotC* as anchoring motifs, and we investigated whether *B. mori* vaccinated with the recombinant spores could escape from the attack of *Bm*NPV. The results indicated that, after challenging with *Bm*NPV, *B. mori* larvae induced with recombinant spores (10^4 spores) showed a significant lower

cumulative infections compared to *B. mori* larvae induced with wild spores or blank control. It suggested that engineered spores may effectively deliver GP64 antigens to the host cell and induce an unknown adaptive resistance to *Bm*NPV. In addition, *Bm*NPV GP64 is a viral-encoded major envelope glycoprotein involved in host receptor-binding and fusion with the host cell membrane during viral entry. Therefore, it is possible that the competitive binding with the receptor of *B. mori* columnar epithelial cell between the displayed GP64 on recombinant spores surface and GP64 from *Bm*NPV, which could block or diminish the attack on *B. mori* from *Bm*NPV.

In conclusion, this study was designed to support the recent development of *B. subtilis* spores for heterologous antigen presentation and spore-based vaccine. Our results showed that *B. subtilis* spores have the potential for heterologous antigen presentation and ultimately for use as a vaccine system.

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References

1. Barrett JW, Brownwright AJ, Primavera MJ, Palli SR (1998) Studies of the nucleopolyhedrovirus infection process in insects by using the green fluorescence protein as a reporter. J Virol 72:3377–3382
2. Theilmann DA, Blissard GW, Onning B, Ehle JA, Reilly DR, Ohrmann GF, Hiem S, Lak JM (2005) Baculoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy-eighth report of the international committee on taxonomy of viruses. Springer, New York, pp 1129–1185
3. Oomens AG, Monsma SA, Blissard GW (1995) The baculovirus GP64 envelope fusion protein: synthesis, oligomerization, and processing. Virology 209:592–603
4. Kadlec J, Loureiro S, Abrescia NG, Stuart DI, Jones IM (2008) The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. Nat Struct Mol Biol 15:1024–1030
5. Blissard GW, Wenz JR (1992) Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J Virol 66:6829–6835
6. Rahman MM, Gopinathan KP (2003) Characterization of the gene encoding the envelope fusion glycoprotein GP64 from *Bombyx mori* nucleopolyhedrovirus. Virus Res 94:45–57
7. Zhou J, Blissard GW (2008) Identification of a GP64 subdomain involved in receptor binding by budded virions of the baculovirus *Autographica californica* multicapsid nucleopolyhedrovirus. J Virol 82:4449–4460
8. Huang JM, La Ragione RM, Cooley WA, Todryk S, Cutting SM (2008) Cytoplasmic delivery of antigens, by *Bacillus subtilis* enhances Th1 responses. Vaccine 26:6043–6052
9. Kim J, Schumann W (2009) Display of proteins on *Bacillus subtilis* endospores. Cell Mol Life Sci 66:3127–3136
10. Cutting SM, Vander-Horn PB (1990) Genetic analysis. In: Harwood CR, Cutting SM (eds) Molecular biological methods for *Bacillus*. Wiley, Chichester, pp 27–74
11. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
12. Nicholson WL, Setlow P (1990) Sporulation, germination and outgrowth. In: Harwood CR, Cutting SM (eds) Molecular biological methods for *Bacillus*. Wiley, Chichester, pp 391–450
13. Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, Oggioni MR, De FM, Pozzi G, Ricca E (2001) Surface display of recombinant proteins on *Bacillus subtilis* spores. J Bacteriol 183:6294–6301
14. Duc IH, Hong HA, Fairweather N, Ricca E, Cutting SM (2003) Bacterial spores as vaccine vehicles. Infect Immun 71:2810–2818
15. Mauriello EM, Duc IH, Isticato R, Cangiano G, Hong HA, De FM, Ricca E, Cutting SM (2004) Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. Vaccine 22:1177–1187
16. Hoang TH, Hong HA, Clark GC, Titball RW, Cutting SM (2008) Recombinant *Bacillus subtilis* expressing the *Clostridium perfringens* alpha toxoid is a candidate orally delivered vaccine against necrotic enteritis. Infect Immun 76:5257–5265
17. Witteveldt J, Cifuentes CC, Vlak JM, van Hulten MC (2004) Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. J Virol 78:2057–2061
18. Namikoshia A, Wua J, Yamashitaa T, Nishizawab T, Nishiokac T, Arimotoc M, Muroga K (2004) Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquaculture 229:25–35
19. Roekring S, Flegel TW, Malasit P, Kittayapong P (2006) Challenging successive mosquito generations with a densonucleosis virus yields progressive survival improvement but persistent, innocuous infections. Dev Comp Immunol 30:878–892