Surface display of recombinant protein on the cell surface of *Bacillus subtilis* by the CotB anchor protein

Meimei Han · Keiichi Enomoto

Received: 25 February 2010 / Accepted: 21 June 2010 / Published online: 3 July 2010

© Springer Science+Business Media B.V. 2010

Abstract  We developed a novel surface display system based on the CotB anchoring motif in order to express foreign protein on the surface of vegetative *Bacillus subtilis* cells. CotB is a protein in the *B. subtilis* spore coat. In this system, three repeats of the immunodominant ovalbumin T-cell epitope (OVA 323–339) were linked with the cholera toxin B subunit (CTB) to construct a fusion protein, CTB-OVA epi, which was then fused to the C-terminal of the CotB protein so that CTB-OVA epi was expressed in vegetatively-growing *B. subtilis*. The expression and localization of the CTB-OVA epi protein was confirmed by western blotting, immunofluorescence microscopy, and flow cytometry. The results indicated that a CotB-based surface display system was successfully used to express the CTB-OVA epi protein on the surface of vegetative *B. subtilis* cells.

Keywords  *Bacillus subtilis* · Surface display · Expression · CotB protein

Introduction

The heterologous display of peptides and proteins such as antigens, enzymes and receptors on the surface of live bacterial cells is of great value for various biotechnological and industrial applications such as oral vaccine development (Liljeqvist et al. 1997; Lee et al. 2000; Ricci et al. 2000), whole-cell biocatalysts and bioadsorbents (Richins et al. 1997; Catherine et al. 2002; Xu and Lee 1999), combinatorial library screening (Boder and Wittrup 1997), and antibody production (Martineau et al. 1991). More recently, vaccine delivery systems have been developed using the surface display of foreign antigens on live bacterial surfaces, and these are able to provide better levels of immunity against pathogens (Hansson et al. 2001). When a heterogeneous immunogen is expressed on the surface of non-pathogenic bacteria and then orally administered alive, long-lasting immune responses can be elicited (Hansson et al. 2001). The surface structures of the bacteria that act as adjuvants might explain the strong antigenicity of the surface-expressed foreign protein (Hansson et al. 2001).

In Gram-negative bacteria, heterologous surface display has been widely used and is now becoming a promising research area. *E. coli* plays a major role as an expression host that can display heterologous proteins on the cell surface (Jose et al. 2002; Narita et al. 2006). However, there have been few reports on the use of heterologous surface display on Gram-negative bacteria for the purpose of producing edible vaccine vehicles. Compared with Gram-negative bacteria, Gram-positive bacteria are more rigid because they have a much thicker cell wall. Gram-positive bacteria also lack an outer membrane so the secretion of heterologous proteins is correspondingly simple (Medaglini et al. 2001). Therefore, Gram-positive bacteria are considered as good candidates for the development of recombinant live vaccines using a surface display expression system. Furthermore, some Gram-positive bacteria are non-pathogenic or food-grade, and can thus safely be used to generate live bacterial vaccines. For instance, *Streptococcus gordonii* has been employed to
display the tetanus toxin fragment C (TTFC) on the surface of bacteria (Medaglini et al. 2001). Lactobacillus has also been employed to express the severe acute respiratory syndrome (SARS) coronavirus spike protein and human papillomavirus antigen proteins on the surface of bacteria for the production of edible vaccines (Lee et al. 2006; Poo et al. 2006). In this study, we were interested in developing a cell surface display system based on B. subtilis since it offers several of the advantages mentioned above. In addition, B. subtilis is closely related to an edible bacterium, Bacillus subtilis (natto). CotB has already been employed as an anchoring protein to display the tetanus toxin fragment C (TTFC) of Clostridium tetani on the surface of B. subtilis spores (Isticato et al. 2001). However, little is known about whether the heterologous protein can be expressed on the surface of vegetative cells of B. subtilis. Therefore, we planned to use B. subtilis as a tool for the production of live recombinant bacteria via the display of a fusion antigen protein on its vegetative cell surface.

CotB is one of the components of the B. subtilis spore coat and has a hydrophilic C-terminal half made up of three 27-amino-acid repeats, which are rich in serine, lysine, and glutamine residues (Ricca and Cutting 2003). Based on analogy to the connective tissue proteins collagen and elastin, the lysine residues in the repeat area are considered to represent the sites of intra- or inter-molecular cross-linking (Ricca and Cutting 2003; Kobayashi et al. 1998). Therefore, we attempted to construct a vaccine delivery vehicle via the display of a foreign antigen on the vegetative cell surface of B. subtilis using CotB as an anchoring motif.

In this study, the ovalbumin (OVA) T-cell epitope (Robertson et al. 2000) was employed as the antigen protein since integration of an ovalbumin T-cell epitope with a MHC II class molecule can induce an OVA-specific T-cell response by oral administration. The cholera toxin B subunit (CTB) was employed as a fusion partner because it is a nontoxic and very useful adjuvant and carrier for enhancing the induction of mucosal antibody responses to the linked antigen (Lebens and Holmgren 1994). To improve the antigenicity of the OVA T-cell epitope, three immunodominant OVA T-cell epitopes (OVA 323–339) were linked to construct OVA epi. We attempted to construct an expression vector using the pHY300 plasmid, which can shuttle between E. coli and B. subtilis. CotB-CTB-OVA epi was successfully displayed on the surface of B. subtilis using CotB as an anchoring motif.

To achieve a high level of protein expression, middle wall protein (MWP) promoters (for the transcription of mRNA) derived from Bacillus brevis 47 were employed (Tsuboi et al. 1988). In addition, a suitable ribosome binding site (RBS) (AAAGGAGG) and an optimal 9 bp distance between the RBS and the initiation codon were selected (Ohashi et al. 2003).

In this study, the repeated OVA T-cell epitope (OVA 323–339) gene linked to the CTB gene, and the cotB gene linked to the middle wall protein (MWP) promoters, were inserted into the pHY300PLK shuttle vector to construct the pHY300-mwp-cotB-ctb-ova epi expression vector, which was used to transform B. subtilis strains. Western blotting analysis showed that CotB-CTB-OVA epi was successfully expressed in B. subtilis. The location of the expressed protein on the bacterial surface was confirmed by fluorescence microscopy using the anti-cholera toxin antibody and a secondary antibody labeled with a fluorescent dye. Flow cytometry was used to confirm and quantitatively analyze the cell-surface-displayed CTB-OVA epi, again using the anti-cholera toxin antibody and a secondary antibody labeled with a fluorescent dye. The results indicated that CTB-OVA epi was successfully displayed on the surface of B. subtilis cells by the CotB anchoring protein.

Materials and methods

Bacterial strains, plasmids, growth conditions, and transformation

The pHY300 plasmid was purchased from Takara Bio (Otsu, Japan). E. coli JM109 was used for cloning the recombinant plasmid of pHY300. B. subtilis 168 trpC2 was kindly gifted by Dr. Mitsuhiro Itaya of The Mitsubishi Kagaku Institute of Life Sciences, and was grown in LB medium at 37°C. Tetracycline (10 µg/ml) was used as the selection antibiotic for transformed competent B. subtilis cells.

The transformation of E. coli JM109 with a recombinant plasmid of pHY300 was performed using the CaCl2 method. The transformation of B. subtilis 168 trpC2 with recombinant plasmids of pHY300 was performed using the electroporation method (Stephenson and Jarrett 1991). B. subtilis 168 trpC2, washed with water and resuspended in pre-chilled 30% polyethylene glycol (PEG) 6000, was electroporated in a 2-mm cuvette with a BTX electro cell manipulator 600 M (Gentronics, San Diego, CA) at a voltage of 2.5 kV and a resistance of 186 ohms.

Ampicillin was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Tetracycline was purchased from Wako (Wako, Osaka, Japan).

Construction of fusion antigen gene

The fusion gene of ova epi (ova epit1-ova epi2-ova epi3) was constructed by linking three OVA 323–339 sequences using the overlap extension PCR method. The forward primer OVA-1 and the reverse primer OVA-2 were mixed, denatured at 90°C, reannealed at 58°C and extended by KOD DNA polymerase (Toyobo, Osaka, Japan) at 68°C for
10 cycles, and then the forward primer OVA-3, consisting of the first 15 bp of the 5' end sequence of the primer OVA-1, and the reverse primer OVA-4, consisting of the first 15 bp of the 5' end sequence of the reverse primer OVA-2, were added to amplify the PCR product, namely the ova epi 1-ova epi 2 fusion gene. Then, the purified ova epi 1-ova epi 2 fusion gene and the reverse primer OVA-5, consisting of the first 15 bp of the 5' end sequence of the reverse primer OVA-4 were mixed, and 10 PCR cycles were performed with denaturation at 90°C, annealing at 60°C and extension by KOD polymerase at 68°C. Then the forward primer OVA-3 and the reverse primer OVA-6, consisting of the first 15 bp of the 5' end sequence of the reverse primer OVA-5, were added to amplify the final PCR product, namely the ova epi (ova epi 1-ova epi 2-ova epi 3) fusion antigen gene.

The C-terminus ending of CTB was fused to OVA T-cell epitopes via a KRWLV linker (Fig. 1). The CTB gene (ctb) amplified from the genome of Vibrio cholerae strain 569B was kindly gifted by Professor Takeshi Honda of Osaka University. The strain 569B was kindly gifted by Professor Takeshi Honda of Osaka University. The genome of Bacillus brevis was constructed using the overlap extension PCR method. The forward primer OVA-1, and the reverse primer OVA-4, consisting of the first 15 bp of the 5' end sequence of the MWP promoters, were used to amplify the PCR product mwp-RBS from the genome of B. subtilis (Ohashi et al. 2003). This sequence was based on experimental data showing that the optimal RBS was AAAGGAGG and the optimal distance between the RBS and start codon was 7–9 nucleotides (Ohashi et al. 2003). The forward primer MWP-Fw and the reverse primer MWP-RBS-Rv, which is complementary to the 3' end sequence of the MWP promoters, were used to amplify the PCR product, namely the mwp-RBS-cotB fusion gene (containing 15 bp of the 5' end sequence of cotB).

To construct a surface display expression vector, a competent promoter, an optimal ribosome binding site (RBS), and a suitable anchoring protein are necessary. In this study, to achieve a high level of protein expression, MWP promoters derived from Bacillus brevis 47 were employed. The consensus RBS sequence (AAAGGAGG) was determined by searching the whole genome sequence of B. subtilis (Ohashi et al. 2003). This sequence was based on experimental data showing that the optimal RBS was AAAGGAGG and the optimal distance between the RBS and start codon was 7–9 nucleotides (Ohashi et al. 2003). The forward primer MWP-Fw and the reverse primer MWP-RBS-Rv, which is complementary to the 3' end sequence of the MWP promoters, were used to amplify the PCR product mwp-RBS from the genome of Bacillus brevis. Using the mwp-RBS fusion gene as the template, the 5' end sequence of the cotB gene was linked to the 3' end of mwp-RBS using the overlap extension PCR method. The forward primer MWP-Fw and the reverse primer MWP-RBS-cotB-Rv, which is complementary to the 5' end sequence of the cotB gene and the 3' end sequence of the MWP promoters, were used to amplify the PCR product, namely the mwp-RBS-cotB fusion gene (containing 15 bp of the 5' end sequence of cotB). Table 2 shows the sequences of the primers used for the construction of mwp-cotB-ctb-ova epi (The sequence underlined is RBS sequence and the sequence indicated in lowercase is the 9-nucleotide-spacing between the RBS and start codon).

To construct the mwp-RBS-cotB fusion gene, mwp-RBS-cotB (containing 15 bp of the 5' end sequence of cotB) was linked to cotB using overlap extension PCR. The cotB gene sequence was amplified from the genome of B. subtilis 168 using the forward primer CotB-Fw and the reverse primer CotB-Rv. The two PCR products, mwp-cotB (containing 15 bp of the 5' end sequence of the cotB gene) and the cotB gene, were mixed, denatured at 90°C, reannealed at 47°C and extended by KOD polymerase at 68°C for 10 cycles, and then the forward primer ctb-Fw and the reverse primer OVA-6 were added to amplify the final PCR product, namely the mwp-cotB fusion gene. Taq DNA polymerase (Takara Bio, Shiga, Japan) was used in this PCR to add one A nucleotide at the 3' termini of the PCR product to enable ligation with the T vector. All the primers were purchased from Invitrogen Japan (Tokyo, Japan).

The antigen gene, ctb-ova epi, was digested out of the pUC18-ctb-ova epi plasmid with BamHI and Hind III restriction enzymes and then cloned into the pHY300 vector digested at the corresponding sites to form the pHY300-ctb-ova epi recombinant plasmid. The sequence of mwp-RBS-cotB amplified by PCR using Taq polymerase was cloned into the pHY300-ctb-ova epi recombinant plasmid using the T-A cloning method as described below.

![Fig. 1 Structure of the ctb-ova epi fusion antigen gene](image-url)
The T-vector of pHY300-ctb-ova epi was prepared according to the method described above (Ohashi et al. 2003). A 2-μg amount of pHY300-ctb-ova epi was completely digested with 30 U of \( \text{SmaI} \) at 30°C for 6 h and subjected to phenol/chloroform extraction. After precipitation with ethanol, the DNA was dissolved in 10 μl of TE buffer. An aliquot of 5 μl of the digested plasmid solution was treated with Ex Taq DNA polymerase (Takara, Otsu, Japan) at a ratio of 2.5 U/μg plasmid in 50 μl buffer solution (1×Ex Taq buffer supplemented with 2 mM dTTP) at 72°C for 2 h. After two phenol/chloroform extractions and isopropanol precipitation, the T-vector was dissolved in 10 μl of TE buffer and stored at -80°C until use. Then, the sequence of MWP-RBS-cotB amplified by PCR using Taq polymerase was introduced into the T-vector at 16°C overnight using a DNA Ligation Kit (Takara, Otsu, Japan) according to the manufacturer’s instructions. Figure 2 shows the structure of the pHY300-cotB-ctb-ova epi expression plasmid for cell surface display.

The DNA sequence of the plasmid insert was checked using the dye-terminator method of Bio Matrix Research (Nagareyama, Japan).

Expression of antigen protein in \( B. \ subtilis \)

A single positive colony of \( B. \ subtilis \) 168 \( \text{trpC2} \) transformed by pHY300-ctb-ova epi or pHY300-cotB-ctb-ova epi was grown in 5 ml of LB medium supplemented with tetracycline (10 μg/ml) under shaking at 37°C for 8 h. Bacteria were pelleted by centrifugation. Proteins were extracted by adding 50 μl of 2× SDS–PAGE sample buffer and heating for 5 min at 90°C. The suspension was centrifuged and 15 μl of supernatant were applied to SDS–PAGE, followed by western blotting to detect the expression of the antigen peptide.

Western blotting

Proteins were separated on SDS–PAGE gels and then transferred onto PVDF transfer membranes (Amersham Bioscience, Tokyo, Japan) using the semi-dry method (Gravel and Table 1 The primers used for construction of ctb-ova epi

| Primers’ name | Sequence of primer(5′–3′) | Annealing temperature (°C) |
|---------------|---------------------------|----------------------------|
| OVA1          | ATTAGCCAGCGTGCATGCGGGAATTAATGAAGCGGGCCGCAGCATTCGCGGCTGG | 58 |
| OVA2          | AGCCCTGCTCATTGTATTTTGTCATGCGGCGCATGACGAGCCTGGAAATGCGAGAGCAATGCC | 58 |
| OVA3          | ATTAGCCAGGCCGTT | 60 |
| OVA4          | GCGGACCCAGCAGGCCGCTCGCCTCATT | 60 |
| OVA5          | GCGAAGGCTTGCCGAGGCCTCAGT | 60 |
| OVA6          | GCGAAGGCTTGCCGAGGCCTCAGT | 60 |
| CTB-Fw        | GCGGATCCATGACACCCTCATAATTACTG | 47 |
| Linker-Rv     | CACCGCCAGCGTTTATTGCCCATACTAAT | 58 |
| ova-Rv        | CACCGCCTGGCTAAATCACCAGCCAGCAGTTT | 47 |

Table 2 The primers used for construction of mwp-cotB-ctb-ova epi

| Primers’ name | Sequence of primer(5′–3′) | Annealing temperature (°C) |
|---------------|---------------------------|----------------------------|
| MWP-Fw        | AACTTGGCTGTTGAAACTTGGAAATG | 46 |
| MWP-RBS-Rv    | CCTCCCTTCGAGGAAAGCCTCCTC | 46 |
| MWP-RBS-cotB-Rv | CATTCTCCTTGTGCTCATCTCCTTTCGAGGAAG | 52 |
| CotB-Fw       | ATGAGCAAGAGGAAATGAAATCAT | 56 |
| CotB-Rv       | GGATGATTGATCATCTGAGATTTTAG | 46 |

Fig. 2 Construction of the pHY300-cotB-ctb-ova epi expression plasmid for cell surface display
Golaz 1996). Rabbit anti-cholera toxin antibody (C3062) (Sigma–Aldrich Japan, Tokyo, Japan) was used as the primary antibody for CTB-OVA epi antigen proteins. The secondary antibody was donkey anti-rabbit antibody conjugated with horseradish peroxidase (NA 934 V) (Amersham Bioscience, Tokyo, Japan). The signal was detected with enhanced chemiluminescence reagents (Amersham Bioscience, Tokyo, Japan). CTB (Sigma–Aldrich Japan, Tokyo, Japan) was used as the positive control.

Immunofluorescence microscopy

Immunostaining was performed as follows: B. subtilis cells were cultivated in LB medium at 37°C for 8 h, collected by centrifugation at 3,500 × g for 5 min at 4°C, and washed with phosphate-buffered saline (PBS) (pH 7.2). After resuspension in PBS containing 1% bovine serum albumin (BSA) (OD 600 = 1.0) and incubation for 30 min at room temperature, the cells and the primary antibody were incubated in PBS containing 1% BSA for 1 h at room temperature. Rabbit anti-CT (C 3062) (Sigma–Aldrich, Tokyo, Japan) diluted 2000 times was used as the primary antibody. After washing with PBS, the cells were incubated for 1 h at room temperature with the second antibody, a 1:200 dilution ratio of goat anti-rabbit IgG conjugated with FITC (F 0382) (Sigma–Aldrich, Tokyo, Japan). After washing with PBS, the cells were observed by microscopy.

Flow cytometry

One hundred μl of the B. subtilis suspension in PBS with an optical absorbance of 0.02 at 600 nm were stained with the same volume of the 1/2,000 diluted rabbit anti-CTB antibodies (Sigma C3062) for 20 min on ice. Cells were then washed with PBS and stained with 100 μl of FITC-labeled goat anti-rabbit IgG (Sigma F0382) at 1/100 dilution. Flow cytometry was optimized for the analysis of bacteria by raising the FSC (forward scatter) voltage to E03 (FACScan, Becton–Dickinson Japan, Tokyo). A total of 50,000 particles were acquired in a logarithmic fluorescence mode.

Results

Expression of CotB-CTB-OVA epi antigen protein on the cell surface of B. subtilis

To express the CTB-OVA epi antigen protein on the surface of B. subtilis cell, the gene of CTB-OVA epi was fused to the end of the cotB gene to yield the pHY300-cotB-ctb-ova epi recombinant expression plasmid (Fig. 2).

The pHY300-ctb-ova epi recombinant expression plasmid containing the MWP promoter was also constructed as a control of protein expression. These recombinant plasmids were transformed into B. subtilis 168trpC2 by electroporation. B. subtilis 168trpC2 cells harboring pHY300-cotB-ctb-ova epi or pHY300-ctb-ova epi were grown at 37°C in LB medium containing tetracycline (10 μg/ml) to induce expression of the antigen proteins. It is well known that B. subtilis strains can produce a number of extracellular proteases that recognize and degrade heterologous proteins. In this study, we detected the expression status of a heterologous protein after different length incubations (8, 10, 12, 14, 16, and 18 h), and found that after 8 h of incubation, at which point there was little degradation, the expressed antigen protein became increasingly degraded (data not shown). Therefore, we chose the 8 h incubation to avoid proteolysis as much as possible. The expressed fusion proteins were analyzed by western blotting with the anti-cholera toxin antibody used as the primary antibody. Figure 3 shows the results of the western blotting analysis of expressed antigen protein in B. subtilis.

As shown in Fig. 3, a band of 18 kDa corresponding to the theoretical molecular weight of the CTB-OVA epi antigen protein was observed (Fig. 3, lane 3). The 49-kDa band most likely represents the fusion protein of the CTB-OVA epi antigen protein (18 kDa) linked to the CotB surface display protein (31 kDa) (Fig. 3, lane 4). The results of the western blotting analysis showed that the CotB-CTB-OVA epi fusion protein was successfully expressed in B. subtilis.
Immunofluorescence microscopy

Immunofluorescence labeling of cells was performed using the rabbit anti-cholera toxin antibody as a primary antibody and the goat anti-rabbit IgG conjugated with FITC as the secondary antibody. As shown in Fig. 4, the green fluorescence of the immunostained CTB-OVA fusion protein was observed in *B. subtilis* 168 cells harboring the pH300-mwp-cotB-ctb-ova epi plasmid (Fig. 4–4), whereas cells harboring the control plasmid pH300-mwp-ctb-ova epi were not immunostained (Fig. 41), indicating that CTB-OVA was displayed on the cell surface of *B. subtilis* via the CotB anchor protein.

Flow cytometric analysis

Flow cytometry was used to quantitatively analyze the cell surface display of CTB-OVA epi. The cell surface-displayed CTB-OVA epi was stained with the rabbit anti-cholera toxin antibody as the primary antibody and goat anti-rabbit IgG conjugated with FITC as the secondary antibody, and *B. subtilis* cells harboring the plasmid pH300-ctb-ova epi were used as a control. Cells displaying CotB-CTB-OVA epi showed significantly more intense fluorescence signals than the control cells (Fig. 5). In the absence of the primary antibody, the fluorescence profile of the negative control was almost the same as that in the presence of the primary antibody (data not shown). Therefore, the fluorescence from the negative control was due to the non-specific binding of the FITC-conjugated secondary antibody. This result is consistent with the data shown in Fig. 4, and together, the results indicate successful cell surface display of the CTB-OVA epi antigen.

Discussion

We report here the use of a novel, CotB-based surface display system to express the CTB-OVA epi antigen protein on the surface of vegetative cells of *B. subtilis*. It has previously been reported that at least 20 polypeptides are organized to form the two layers of the *B. subtilis* spore coat (Driks 1999; Henriques and Moran 2000). Some of these polypeptides have been associated with the outer part of the coat, but for all of them the exact location within the coat and the protein domain required for surface exposure are not known (Zheng et al. 1988). However, the proteinaceous nature of this multilayered coat suggests the possibility of using its structural components as fusion partners for the expression of heterologous proteins on the spore surface (Isticato et al. 2001). The CotB protein was used as an anchoring motif to express the tetanus toxin fragment C (TTC) of *Clostridium tetani* on the surface of *B. subtilis* spores (Isticato et al. 2001) by integrating the cotB gene...
and TTFC gene into the \textit{B. subtilis} genome. The TTFC was successfully displayed on the surface of \textit{B. subtilis} spores by the CotB coat protein, which was expressed in the outer layer of the \textit{B. subtilis} spore coat (Isticato et al. 2001). However, the procedure of gene integration was complex and time-consuming and the incubation period for sporulation usually required 24 h. In addition, the spores also needed to be washed and purified (Isticato et al. 2001). In this study, we hypothesized that CotB could be employed as an anchoring motif to display the antigen protein on the surface of vegetative cells of \textit{B. subtilis} directly, thus simplifying the procedure for protein expression. \textit{B. subtilis} is non-pathogenic, and is closely related to an edible bacterium, \textit{Bacillus subtilis} (natto), which is used to produce fermented soybean “natto” (Itaya and Matsui 1999; Qiu et al. 2004). Therefore, the potential for development of vaccine delivery systems using \textit{B. subtilis} is significant and promising.

In this study, we constructed a recombinant expression vector based on the pHY300 vector by inserting the \textit{cotB} and CTB-OVA epi genes into this plasmid. The expression of the antigen proteins was only induced after the recombinant \textit{B. subtilis} strains were grown in LB medium at 37°C for 8 h, without requiring other induction factors. This procedure simplified the preparation of the surface displayed bacterial vaccine to a great extent. However, the expressed CTB-OVA epi antigen protein on the cell surface of \textit{B. subtilis} was degraded over time, probably due to proteases produced by \textit{B. subtilis}. The amount of CTB-OVA epi protein expressed by this surface display system was not particularly high, according to the difference in fluorescence intensity between cells displaying CTB-OVA epi and the negative control. However, this functional CotB-based surface display system for the vegetative cells of \textit{B. subtilis} is not only able to provide better levels of immunity against pathogens than the ordinary system but also establishes a good foundation for using \textit{B. subtilis} (natto) as a organism for the future production of edible vaccine vehicles. Here we have described the expression of an antigen protein from a gene in the genome of \textit{B. subtilis}. In future experiments, the CotB-CTB-OVA epi gene will be integrated into the genome of \textit{B. subtilis} for the stability of the antigen gene. Further work to increase the yield and examine the immunity of the displayed protein on the surface of \textit{B. subtilis} cells is still required.

Acknowledgments We thank Dr. Mitsuhiro Itaya of The Mitsubishi Kagaku Institute of Life Science for providing \textit{B. subtilis} strains. We also thank Professor Takeshi Honda of Osaka University for providing the CTB gene construct. We express our sincere gratitude to Professor Keiko Udaka of Kochi University for allowing us to do the flow cytometry experiment and valuable discussion.



References

Boder ET, Wittrup KD (1997) Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 15:553–557

Catherine MHC, Mulchandani A, Chen W (2002) Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. Appl Environ Microbiol 68:2026–2030

Driks A (1999) \textit{Bacillus subtilis} Spore Coat. Microbiol Mol Biol Rev 63:1–20

Gravel P, Golaz O (1996) Protein blotting by semidy method. In: Walker JM (ed) The protein protocols handbook. Humana Press. New Jersey, USA, pp 249–260, ISBN 9780896033382

Hansson M, Samuelson P, Gunneriusson E, Stahl S (2001) Surface display on Gram positive bacteria. Comb Chem High Throughput Screen 4:171–184

Henriques A, Moran CP (2000) Structure and assembly of the bacterial endospore coat. Methods 20:95–110

Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, Oggianni MR, Felice MD, Pozzi G, Ricca E (2001) Surface display of recombinant proteins on \textit{Bacillus subtilis} spores. J Bacteriol 183:6294–6301

Itaya M, Matsui K (1999) Conversion of \textit{Bacillus subtilis} 168: \textit{Natto} producing \textit{Bacillus subtilis} with mosaic genomes. Biosci Biotechnol Biochem 63:2034–2037

Jose J, Bernhardt R, Hannemann F (2002) Cellular surface display of dimeric Adx and whole cell P450-mediated steroid synthesis on \textit{E. coli}. J Bacteriol 95:257–268

Kobayashi K, Suzuki SI, Izawa Y, Miwa K, Yamanaka S (1998) Transglutaminase in sporulating cells of \textit{Bacillus subtilis}. J Gen Appl Microbiol 44:85–91

Lebens M, Holmgren J (1994) Mucosal vaccines based on the use of cholera toxin B subunit as immunogen and antigen carrier. Dev Biol Stand 82:215–227

Lee JS, Shin KS, Pan JG, Kim CJ (2000) Surface-displayed viral antigens on \textit{Salmonella} carrier vaccine. Nat Biotechnol 18:645–648

Lee JS, Poo H, Han DP, Hong SP, Kim K, Cho MW, Kim E, Sung MH, Kim CJ (2006) Mucosal Immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on \textit{Lactobacillus casei} induces neutralizing antibodies in mice. J Virol 80:4079–4087

Liljeqvist S, Samuelson P, Hansson M, Nguyen TN, Binz H, Stahl S (1997) Surface display of the cholera toxin B subunit on \textit{Staphylococcus xylosus} and \textit{Staphylococcus carnosus}. Appl Environ Microbiol 63:2481–2488

Martineau P, Charbit A, Leclerc C, Werts C, Callaghan DO, Hofnung M (1991) A genetic system to elicit and monitor anti-Peptide antibodies without peptide synthesis. Biotechnology (NY) 9:170–172

Medaglini D, Ciabattini A, Spinosa MR, Maggi T, Marocca H, Oggianni MR, Pozzi G (2001) Immunization with recombinant \textit{Streptococcus gordonii} expressing tetanus toxin fragment C confers protection from lethal challenge in mice. Vaccine 19:1931–1939

Narita J, Okano K, Tateno T, Tanino T, Sewaki T, Sugn MH, Fukuda H, Kondo A (2006) Display of active enzymes on the cell surface of \textit{Escherichia coli} using PgsA anchor protein and their application to bioconversion. Appl Microbiol Biotechnol 70:564–572

Ohashi Y, Ohshima H, Tsuge K, Itaya M (2003) Far different levels of gene expression provided by an oriented cloning system in \textit{Bacillus subtilis} and \textit{Escherichia coli}. FEBS Microbiol Lett 221:125–130
Poo H, Pyo HM, Lee TY, Yoon SW, Lee JS, Kim CJ, Sung MH, Lee SH (2006) Oral administration of human papillomavirus type 16 E7 displayed on Lactobacillus casei induces E7-specific antitumor effects in C57/BL6 mice. Int J Cancer 119:1702–1709

Qiu D, Fujita K, Sakuma Y, Tanaka T, Ohashi Y, Oshima H, Tomita M, Itaya M (2004) Comparative analysis of physical maps of four Bacillus subtilis (natto) genomes. Appl Environ Microbiol 70: 6247–6256

Ricca E, Cutting SM (2003) Emerging applications of bacterial spores in nanobiotechnology. J Nanobiotechnol 1:1–10

Ricci S, Medaglini D, Rush CM, Marcello A, Peppoloni S, Manganelli R, Palu G, Pozzi G (2000) Immunogenicity of the B Monomer of Escherichia coli Heat-Labile Toxin Expressed on the Surface of Streptococcus gordonii. Infect Immun 68:760–766

Richins RD, Kaneva I, Mulchandani A, Che W (1997) Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase. Nat Biotechnol 15:984–987

Robertson M, Jensen PE, Evavold BD (2000) DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323–339 epitope. J Immunol 164:4706–4712

Stephenson M, Jarrett P (1991) Transformation of Bacillus subtilis by electroporation. Biotechnol Tech 5:9–12

Tsuboi A, Uchihi R, Adachi T, Sasaki T, Hayakawa S, Yamagata H, Tsukagoshi N, Udaka S (1988) Characterization of the genes for the hexagonally arranged surface layer proteins in protein-producing Bacillus brevis 47: complete nucleotide sequence of the middle wall protein gene. J Bacteriol 170:935–945

Xu Z, Lee SY (1999) Display of polyhistidine peptides on the Escherichia coli cell surface by using outer membrane protein C as an anchoring motif. Appl Environ Microbiol 65:5142–5147

Zheng L, Donovan WP, Fitz-James PC, Losick R (1988) Gene encoding a morphogenic protein required in the assembly of the outer coat of the Bacillus subtilis endospore. Genes Dev 2:1047–1054