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SHORT COMMUNICATION

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

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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

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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 intergration 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 pHY300PLK plasmid, which can shuttle between *E. coli* and *B. subtilis*. This expression vector can be manipulated in *E. coli* because it has a higher transformation efficiency in *E. coli* than in *B. subtilis*.

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 *trp*C2 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 $CaCl_2$ 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 epi1-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 *ctb* gene was used as the template for the first PCR. The linker sequence was linked to *ctb* by PCR using the forward primer ctb-Fw complementary to the 5′ end sequence of *ctb* and the reverse primer linker-Rv. KOD-plus DNA polymerase was used to amplify the *ctb-linker* fusion gene. Then, using the *ctb-linker* fusion gene as the template, PCR was performed to link the 5′ end sequence of *ova epi* to the 3′ end of the *ctb-linker* gene. The forward primer ctb-Fw and the reverse primer linker-ova-Rv, which is complementary to the 3′ end sequence of *ctb-linker* and consists of the first 15 bp of the 5′ end sequence of *ova epi* were used. Finally, *ctb-linker-ova epi* was constructed using the overlap extension PCR method. The two PCR products, *ctb-linker-ova-epi* (containing 15 bp of the 5′ end sequence of *ova epi*) and *ova epi,* 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 OVA6 were added to amplify the final PCR product, namely the *ctb-ova epi* fusion antigen gene. All the PCR products were extracted and purified using a Gel Extraction Kit (Qiagen, Tokyo, Japan). All the primers were purchased from Invitrogen Japan (Tokyo, Japan). Table 1 shows the sequences of the primers and the annealing temperatures for the PCR steps (The sequence underlined is the restriction endonuclease site).

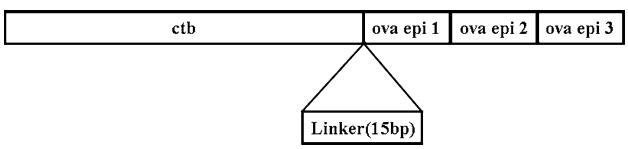

Fig. 1 Structure of the *ctb-ova epi* fusion antigen gene

Construction of recombinant plasmid 9

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*-RBS-*cotB* (containing 15 bp of 5′ end sequence of the *cotB* gene) and the *cotB* gene, were mixed, denatured at 90°C, reannealed at 46°C and extended by KOD polymerase at 68°C for 10 cycles, then the forward primer MWP-Fw and the reverse primer CotB-Rv were added to amplify the final PCR product, namely the *mwp*-RBS-*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 *Bam*HI 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.

Table 1 The primers used for construction of *ctb-ova epi*

Primers' name	Sequence of primer(5′–3′)	Annealing temperature (°C)
OVA1	ATTAGCCAGGCGGTGCATGCGGCGCATGCGGAAATTAATGAAGCGGGCCGCAGCATTCGCGGCTGG	58
OVA2	ACGCCCTGCCTCATTGATTTCGGCATGCGCTGCATGGACG GCTTGAGAGA TCCAGCCGCGAATGCT	58
OVA3	ATTAGCCAGGCGGTG	60
OVA4	GCGAGACCAAATGCCACGCCCTGCCTCATT	60
OVA5	GCGACCGGCCTCGTTGATTTCTGCATGCGCGGCATGAACT GCCTGGGAAATGCGAGACCAAATGCC	60
OVA6	GCGAAGCTTGCGACCGGCCTCGTT	60
CTB-Fw	GCGGGATCCATGACACCTCAAAATATTACTGAT	47
Linker-Rv	CACCAGCCAGCGTTTATTTGCCATACTAAT	58
ova-Rv	CACCGCCTGGCTAATCACCAGCCAGCGTTT	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	AACTTGGCTGTTGTAAACTTTGAAAATGC	46
MWP-RBS-Rv	CCTCCTTTCGCAGGAAAGCCTCGCTTTTTG	46
MWP-RBS-cotB-Rv	CATTCTCCTCTTGCTCAT**gatcaatttCCTCCTTT**CGCAGGAAAG	52
CotB-Fw	ATGAGCAAGAGGAGAATGAAATATCAT	56
CotB-Rv	GGATGATTGATCATCTGAAGATTTTAG	46

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 *sam* I 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 display expression plasmid.

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 *trp*C2 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.

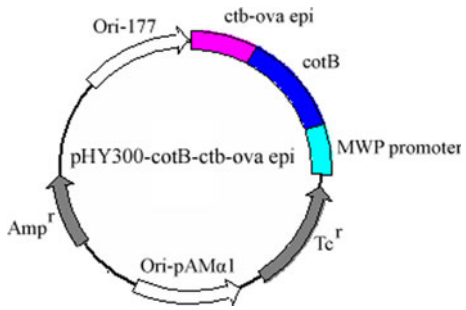

Fig. 2 Construction of the pHY300-cotB-ctb-ova epi expression plasmid for cell surface display

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

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. Immunofluorescence microscopy analysis was performed using an Olympus IX70 Inverted epi-fluorescence microscope (Olympus, Tokyo, Japan).

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* 168*trpC2* by electroporation. *B. subtilis* 168*trpC2* 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.*

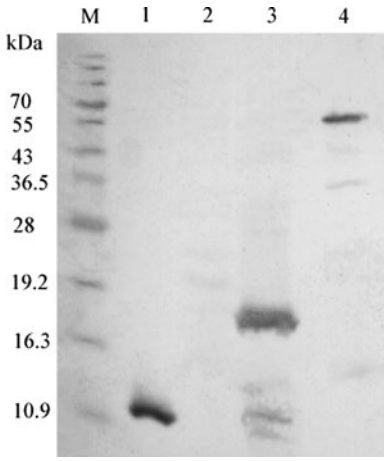

Fig. 3 Western blotting analysis of the antigen protein. Anti-CT antibody was used. Lane M, prestained size marker of protein. *Lane 1*, CTB (molecular weight = 11 KDa). *Lane 2*, *B. subtilis* 168 (negative control). *Lane 3*, *B. subtilis* 168 harboring the pHY300-ctb-ova epi plasmid. *Lane 4*, *B. subtilis* 168 harboring the pHY300-cotB-ctb-ova epi plasmid

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 pHY300-mwp-cotB-ctb-ova epi plasmid (Fig. 4*2–4*), whereas cells harboring the control plasmid pHY300-mwp-ctb-ova epi were not immunostained (Fig. 4*1*), 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 pHY300-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.

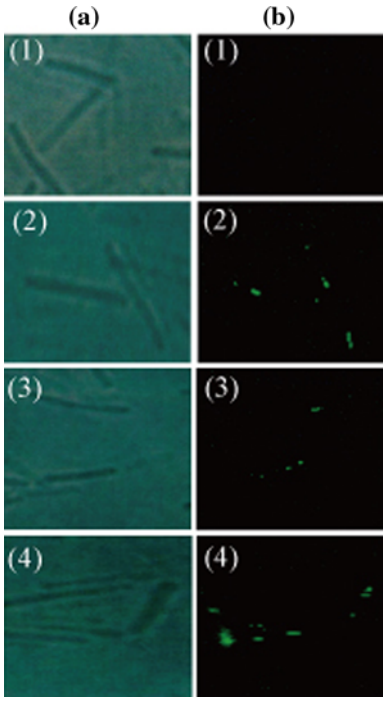

Fig. 4 Immunofluorescence labeling of transformed cells. (*1*) *B. subtilis* 168 cells harboring the pHY300-ctb-ova epi plasmid were grown at 37°C for 8 h in LB medium (negative control). (*2–4*) *B. subtilis* 168 cells harboring the pHY300-cotB-ctb-ova epi plasmid were grown at 37°C for 8 h in LB medium. Cells were labeled with the primary rabbit anti-CT antibody, followed by the secondary goat anti-rabbit IgG conjugated with FITC. Bright-field images (*a*) and the corresponding fluorescence (*b*) micrographs are shown (×1,500)

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 (TTFC) of *Clostridium tetani* on the surface of *B. subtilis* spores (Isticato et al. 2001) by integrating the cotB gene

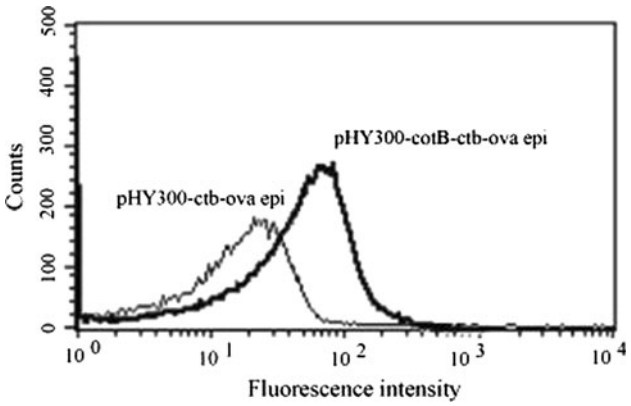

Fig. 5 Flow cytometric analysis of *B. subtilis* harboring the pHY300-ctb-ova epi and pHY300-cotB-ctb-ova epi plasmids. Transformants were grown at 37°C for 8 h in LB medium containing 10 μg/ml of tetracycline. Cells were labeled with the primary rabbit anti-CT antibody, followed by the secondary goat anti-rabbit IgG conjugated with FITC. A total of 50,000 cells were analyzed in each experiment

and TTFC gene into the *B. subtilis* genome. The TTFC was successfully displayed on the surface of *B. subtilis* spores by the CotB coat protein, which was expressed in the outer layer of the *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 *B. subtilis* directly, thus simplifying the procedure for protein expression. *B. subtilis* is non-pathogenic, and is closely related to an edible bacterium, *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 *B. subtilis* is significant and promising.

In this study, we constructed a recombinant expression vector based on the pHY300 vector by inserting the *cotB* and CTB-OVA epi genes into this plasmid. The expression of the antigen proteins was only induced after the recombinant *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 *B. subtilis* was degraded over time, probably due to proteases produced by *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 *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 *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 *B. subtilis*. In future experiments, the CotB-CTB-OVA epi gene will be integrated into the genome of *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 *B. subtilis* cells is still required.

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