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**Expression of Recombinant T-cell Epitopes  
in the Cell and on the Surface of  
*Bacillus subtilis***

**Meimei Han**

Kochi University of Technology

**Dissertation**

Submitted for the Degree of Doctor of Philosophy

Graduate of School of Engineering

Environmental Systems Engineering

Kochi University of Technology, 2009

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# ABSTRACT

## Expression of Recombinant T-cell Epitopes in the Cell and on the Surface of *Bacillus subtilis*

Meimei Han

Production of edible vaccines by plant is a significant innovation since plant-derived vaccines offer the advantages of little risk of infection, low cost, and easy control of production scale. However, the long growth period and the possibility of gene diffusion to surrounding plants by pollination limit the application of antigen-producing transgenic plants. Therefore, the expression of vaccine in edible bacteria sounds attractive because of easy cultivation, short harvest period, and safety.

*Bacillus subtilis* offers several advantages over other Gram-positive bacteria: (i) it is non-pathogenic; (ii) the extensive genetic knowledge has been accumulated. (iii) it has a naturally high secretion capacity. In addition, *B. subtilis* is a bacterium which is closely related to an edible bacterium, *Bacillus subtilis* (natto). Therefore, I am interested to use *B. subtilis* as a tool for edible vaccine. In this study, I attempted to express antigen proteins in the bacterial cells and on the surface of *B. subtilis*.

**Part I** Expression of recombinant allergens of Japanese cedar pollen from a gene integrated in the genome of *B. subtilis*

The gene integrated in the genome of bacteria generally has a high stability, so antibiotics harmful to human being are unnecessary to maintain the antigen gene. Thus, the characterization of the mode of expression of antigens from the gene integrated in bacterial genome is significant for the development of edible vaccine.

In this study, I attempted to produce edible vaccine using *Bacillus subtilis* (natto), an edible bacterium used to produce fermented soybean “natto”. *B. subtilis* (natto) is

an ideal food carrier for edible vaccines for its easy production and the safety of bacterial strain. However, *B. subtilis* (natto) has a low ability of recombination for the integration of exogenous DNA into its genome. To circumvent this problem, I used *B. subtilis* which has the DNA highly homologous to *B. subtilis* (natto) and has an ability to incorporate exogenous DNA into its genome. Furthermore, repeated transformation of a *B. subtilis* strain with genomic DNA from *B. subtilis* (natto) results in a hybrid *B. subtilis* in which a part of *B. subtilis* genome is replaced with the genome of *B. subtilis* (natto). Thus the hybrid bacterium has the ability to ferment natto. Therefore, if we can integrate an antigen gene into the genome of *B. subtilis* and the bacteria having the antigen gene in its genome can express antigen protein, we can use the hybrid bacterium between *B. subtilis* and *B. subtilis* (natto) for the production of edible vaccine. Finally, natto fermented by the hybrid bacteria expressing the antigen protein becomes a useful therapeutic agent.

To control IgE-mediated allergic diseases, immunotherapy based on allergen-specific T cell epitope peptides has been becoming a safe and effective treatment. As one of the serious type I allergic diseases in Japan, Japanese cedar (*Cryptomeria japonica*) pollinosis, which spreads over more than 24% of the Japanese population, is a serious social problem. Cry j 1 and Cry j 2 are two major allergens isolated from the pollen and their multiple domains of T cell epitopes for human being and mouse have been identified. Multiple T-cell epitopes from Cry j 1 and Cry j 2 linked to construct the recombinant peptides have been developed, and basic immunologic studies have exhibited their potency as immunotherapeutic agents. However, the developing and developed immunotherapeutic peptides do not include many T cell epitopes of Cry j 1 and Cry j 2. In former studies of our group, much more epitopes, i.e. five major T-cell epitopes from Cry j 1 and seven from Cry j 2 were selected. In addition to that, these T-cell epitopes did not contained IgE-binding sequences. In former experiments, genes encoding epitopes from Cry j 1 or Cry j 2 were linked and then fused to cholera toxin B subunit (CTB) gene by stepwise PCR to construct two fusion genes, *ctb-cry j 1 epi* and *ctb-cry j 2 epi*. The antigenicities of CTB-Cry j 1 epi and CTB-Cry j 2 epi proteins expressed in *E. coli* were confirmed by

western blotting analysis.

In this study, to examine the mode of expression of antigen protein from the gene in the genome of *B. subtilis*, the *ctb-cry j 1 epi* fusion gene was further linked with the green fluorescent protein (GFP) gene by PCR method to generate *ctb-cry j 1 epi-gfp* gene. The *ctb-cry j 1 epi-gfp* fusion antigen gene was firstly cloned into pHASH120 vector by T-A cloning method and then inserted into the genome of *B. subtilis* BEST2131 by homologous recombination. The expression of antigen protein from the gene in the genome of *B. subtilis* was confirmed by detecting the fluorescence from the antigen protein. Fluorescence from the colonies of *B. subtilis* was measured by a CCD camera under blue LED light at 470nm. *B. subtilis* BEST2131-*ctb-cry j 1 epi-gfp* showed green fluorescence but *B. subtilis* BEST20038 (negative control) showed no fluorescence. Fluorescence intensities at 509 nm of *B. subtilis* BEST2131-*ctb-cry j 1 epi-gfp* and *B. subtilis* BEST20038 (negative control) were monitored by a fluorescence spectrophotometer. *B. subtilis* BEST2131-*ctb-cry j 1 epi-gfp* evidently showed an emission peak at 509 nm which is in accordance with the emission peak of GFP. *B. subtilis* BEST20038 showed no peak at 509nm. The fluorescent characteristics of the bacteria demonstrated that the CTB-Cry j 1 epi-GFP antigen protein was successfully expressed from the gene in the genome of *B. subtilis*.

Western blotting analysis was also performed to confirm the optimal expression time of CTB-Cry j 1 epi-GFP antigen protein. A maximal amount of expressed protein corresponding to the theoretical molecular weight of CTB-Cry j 1 epi-GFP (46 kDa) was observed at 10 h in accordance with the maximal fluorescence at 10 h. After 10 h, fluorescence decreased gradually probably due to the proteolysis of the antigen protein. The genomically-integrated transgene produced protein within 10 h, and the production scale can be easily controlled. Western blotting analysis also indicated that the yield of CTB-Cry j1 epi-GFP antigen protein expressed from the gene in the genome of *B. subtilis* is about 0.06mg protein/L of culture and the yield of CTB-Cry j1 epi-GFP antigen protein expressed from the pGETS103-*ctb-cry j 1 epi-gfp* plasmid in *B. subtilis* is about 0.43mg protein/L of culture. The yield of plasmid-expressed antigen was approximately seven-fold greater than genomically-expressed antigen,

which represented 0.01 % of total bacterial protein.

Further investigation on the increase of the yield of expressed protein from the gene in the genome of *B. subtilis* is still required. The complex regulatory mechanisms of the expression of foreign proteins should be investigated.

## **Part II** Expression of fusion protein containing ovalbumin T-cell epitopes on *B. subtilis*

The development of vaccine delivery systems using heterologous display of proteins or peptides on the surface of bacteria is a useful research tool because it can provide better levels of immunity against pathogens. When a heterologous immunogen was expressed on the surface of non-pathogenic bacteria and then orally administered alive, as a result, a long-lasting immune response compared with that of the ordinary vaccine can be elicited. The surface structures of the bacteria which act as adjuvants might explain its strong antigenicity of the surface-expressed foreign protein. Therefore, surface expression system using non-pathogenic bacteria to produce recombinant vaccine sounds attractive.

An ovalbumin (OVA) T cell epitope integrating with MHC II class molecule can be recognized by a transgenic mouse having a specific T cell receptor for the ovalbumin epitope so that it can induce OVA-specific T-cell response by oral administration. Cholera toxin B subunit (CTB) is a nontoxic and very useful adjuvant and carrier to enhance the induction of mucosal antibody responses to the linked antigen. CotB, a protein component of the *B. subtilis* spore coat was employed as a fusion partner to display the antigen gene on the surface of *B. subtilis*. Considering the lower plasmid copy number in *B. subtilis* compared with *E. coli*, I attempted to construct an expression vector using pHY300PLK plasmid which can shuttle between *E. coli* and *B. subtilis*. To get the high level protein expression, middle wall protein (MWP) promoters derived from *Bacillus brevis* 47, powerful multiple promoters for the transcription of mRNA, were employed.

In this study, repeated immunodominant ovalbumin T cell epitope (OVA<sub>323-339</sub>) linked to cholera toxin B subunit (CTB) gene and CotB gene linked to the middle wall

protein (MWP) promoters sequence were inserted into pHY300PLK shuttle vector respectively to construct pHY300-mwp-cotB-ctb-ova epi expression vector.

The result of western blotting analysis displayed that CotB-CTB-OVA epi was successfully expressed in *B. subtilis*. The location of expressed protein on the surface of bacteria was confirmed by fluorescent microscopy using anti-cholera toxin antibody and the secondary antibody labeled with a fluorescent dye. Additionally, flow cytometry was used to confirm and quantitatively analyze the cell-surface-displayed CTB-OVA epi using anti-cholera toxin antibody and the secondary antibody labeled with a fluorescent dye. The results indicated that a Cot B-based surface display system was successfully used to express the CTB-OVA epi antigen on the surface of vegetative cells of *B. subtilis* for safe, effective delivery. This result also demonstrates that Cot B is a competent anchoring motif for the display of antigens not only on the surface of *B. subtilis* spore but also on the surface of *B. subtilis* cells. Therefore, using CotB as an anchoring motif to display antigens on the vegetative cell surface of *B. subtilis*, the preparation process of live bacterial vaccine could be simplified to a great extent. However, further investigation on the increase of the yield of displayed protein on the surface of *B. subtilis* cells is still required.

**Keywords:** *Bacillus subtilis* genome, Japanese cedar pollinosis, Cry j 1, Surface display system, CotB anchoring protein

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# CHAPTER 1

## General Introduction

Vaccines play an important role for preventive medicine for both humans and animals. However, the cost of production and distribution of vaccine limited their application. Compared with traditional vaccine, expression of antibodies against antigens of pathogens in transgenic plants is a significant innovation since plant-derived vaccines offer the advantages of little risk of infection, low cost, and easy control of production scale. Mason *et al.* [1, 2] were the first to use transgenic tobacco to express hepatitis B surface antigen, and to use transgenic tobacco and potato to express the Norwalk virus capsid protein. Takaiwa and colleagues [3-5] generated transgenic rice plants to express recombinant Cry j 1 allergens in seeds for the treatment of Japanese cedar pollinosis. However, the long growth period and the possibility of gene diffusion to surrounding plants by pollination limit the application of antigen-producing transgenic plants. Therefore, expression of vaccines in edible bacteria is an attractive alternative because of their easy cultivation and rapid harvesting time.

*Bacillus subtilis* is a Gram-positive, rod-shaped bacterium commonly found in soil [6]. *B. subtilis* have been employed as a host not only in the industrial production of enzymes also for the expression of foreign proteins with pharmacological or immunological activities [6, 7]. Ferreira et al. [8] reported the advantages of *B. subtilis* over other Gram-positive bacteria: (i) it has a well-established safety record and is considered as a GRAS organism (generally regarded as safe); (ii) it lacks an outer membrane so that it is capable of secreting extracellular proteins directly into the culture medium, which reduces the costs of purification steps; (iii) a large body of knowledge on genetics and physiology of *B. subtilis* has been acquired; (iv) it has a non-biased codon usage and also has the ability to grow in simple and non-expensive media at rapid growth rates; (v) it has the ability to produce spores which is the most

resistant life form. Based on these advantages, more recently, *B. subtilis* becomes a good candidate for the expression of heterologous proteins in vaccine development [9]. Therefore, in this study, I am interested to employ *B. subtilis* as an expression organism for the production of edible vaccine.

Firstly, Chapter 2 describes the expression of fused antigen protein from the gene in the genome of *B. subtilis*.

As we know, the gene integrated in the genome of bacteria generally has a high stability, so antibiotics harmful to human being are unnecessary to maintain the antigen gene. Thus, the characterization of the mode of expression of antigens from the gene integrated in bacterial genome is significant for the development of edible vaccine. In this study, I attempted to produce edible vaccine using *Bacillus subtilis* (natto), an edible bacterium used to produce fermented soybean “natto”. Natto is a traditional Japanese food and an ideal food carrier for edible vaccines because of its easy production and the safety of this bacterial strain. However, it is difficult to integrate exogenous DNA into the genome of *B. subtilis* (natto). To circumvent this problem, we used the slightly different strain, *B. subtilis*, which has DNA highly homologous to *B. subtilis* (natto), but has the ability to incorporate exogenous DNA into its genome [10, 11]. Ohashi *et al.* [12] employed *B. subtilis* to express green

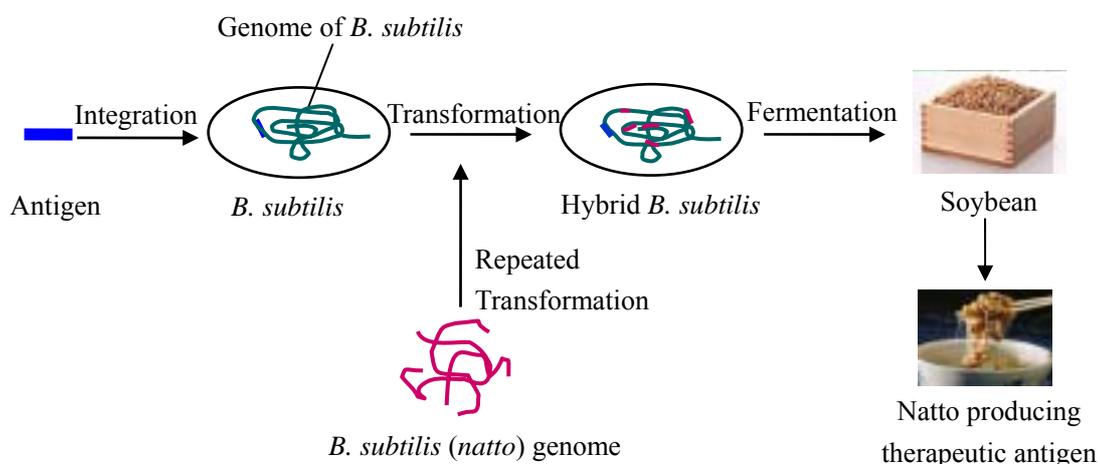


Figure 1.1 Schematic procedure of experiment

fluorescent protein (GFP) from a genomically-integrated gene. Itaya and Matsui [10] showed that repeated transformation of a *B. subtilis* strain with genomic DNA from *B. subtilis* (natto) resulted in a hybrid *B. subtilis* with the ability to ferment natto. Therefore, if I can integrate and express an antigen gene from the genome of *B. subtilis*, I could use the hybrid between *B. subtilis* and *B. subtilis* (natto) for the production of edible vaccine. The natto fermented by these hybrid bacteria would express the antigen and be a useful therapeutic agent. The procedure of experiment described above is shown in **Figure 1.1**.

To control IgE-mediated allergic diseases, immunotherapy based on allergen-specific T cell epitope peptides has become a safe and effective treatment [13-15]. However, a major drawback of this approach is that due to each individual's diversity in MHC class II molecules, people respond to different allergen-derived peptides [16]. Therefore, to achieve sufficient efficacy in a large population of allergic patients, many T-cell allergen epitopes was included [17]. Japanese cedar (*Cryptomeria japonica*) pollinosis, which spreads over more than 24% of the Japanese population, is a serious social problem [18]. Cry j 1 and Cry j 2 are two major allergens isolated from the pollen [19-24] and their multiple domains of T cell epitopes for human being and mouse have been identified [25, 26]. Multiple T-cell epitopes from Cry j 1 and Cry j 2 are linked to construct the recombinant peptides, which have been developed, and basic immunologic studies on the peptides have exhibited their potency as immunotherapeutic agents [17, 26-28]. However, few T-cell epitopes of Cry j 1 and Cry j 2 have thus far been included in the immunotherapeutic peptides. In our study, many more epitopes, that is, five major T-cell epitopes from Cry j 1 and seven from Cry j 2, were used [17, 26].

In this study, cholera toxin B subunit (CTB) was employed as one of fusion partner of Cry j 1 epi antigens because of its special properties. More recently, cholera toxin has become a powerful research tool in microbiology, physiology, cell biology and biochemistry. **Figure 1.2** shows the schematic structure of cholera toxin. The cholera toxin is an oligomeric complex containing an A subunit (CTA), and five pentameric ring of five B subunits. CTA is composed of the toxic domain CTA<sub>1</sub> and a short

sequence CTA<sub>2</sub>. The A subunit is noncovalently linked with B subunits by fitting part of CTA<sub>2</sub> within the central space of CTB pentamer. Toxic CTA<sub>1</sub> can cause diarrhea toxin but CTB is nontoxic and it also can mediate the binding of the holotoxin to GM<sub>1</sub> ganglioside receptors on mucosal epithelia. CTB is considered to be a very useful tool as an adjuvant and immunogenic carrier for other peptide antigens chemically or genetically coupled to it [29-32]. The strong adjuvant properties of CTB in stimulating immune responses of linked foreign antigens are probably due to some immunomodulating effects of cholera toxin. Indeed, when orally administered, such CTB-coupled fusion antigens evidently potentiated intestinal and extraintestinal IgA immune responses against the antigens [29]. Therefore, I attempted to use CTB as an adjuvant and carrier to improve the mucosal immunogenicity of linked antigens for vaccination purposes in this study.

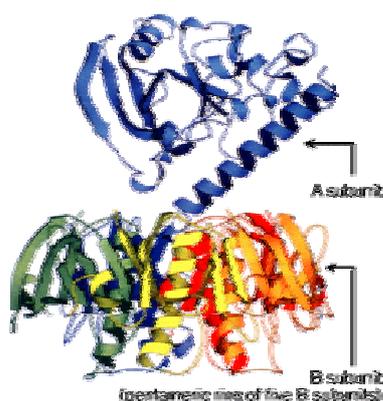


Figure 1.2 Schematic structure of cholera toxin (<http://www-ermm.cbcu.cam.ac.uk>)

As another fusion partner, green fluorescent protein (GFP) isolated originally from the jellyfish *Aequorea victoria* was used to report the expression of Cry j 1 epi antigen protein from the gene in the genome of *B. subtilis* in this study. **Figure 1.3** shows the three-dimensional structure of GFP. GFP has a typical beta barrel structure which contains 11 antiparallel beta strands (width green strip) outside, an alpha-helix inside this beta-structure, and in the center of this beta-structure is the chromophore (white balls) [33]. GFP can fluoresce when it is exposed under blue light [34]. The GFP usually has a major excitation peak at 395 nm and an emission peak at 509 nm. More

recently, GFP is frequently employed as a reporter of protein expression in cell and molecular biology. Inouye *et al.* [35] and Chalfie *et al.* [36] employed GFP as an expression reporter by examining the fluorescence of expressed protein. Therefore, I attempted to use GFP as a reporter of expression in this study. The characterization of the mode of expression of fused antigen protein from the gene in the genome of *B. subtilis* is reported in Chapter 2.

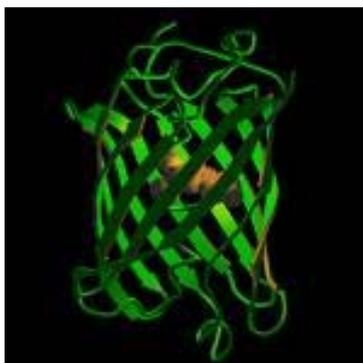


Figure 1.3 Schematic three-dimensional structure of GFP

([http://www.nigms.nih.gov/News/Results/nobel\\_chemistry20081008.htm](http://www.nigms.nih.gov/News/Results/nobel_chemistry20081008.htm))

Secondly, Chapter 3 describes the expression of fusion antigen protein on the surface of *B. subtilis* cell.

Heterologous display of peptide and protein such as antigen, enzyme and receptor on the surface of live bacterial cells is of great value for various biotechnological and industrial applications such as oral vaccine development [37-39], whole-cell biocatalyst and bioadsorbent [40-42], combinatorial library screening [43], and antibody production [44]. More recently, the vaccine delivery systems have been developed using surface display of foreign antigens on the live bacterial surfaces, which are able to provide better levels of immunity against pathogens [45]. When a heterogenous immunogen was expressed on the surface of non-pathogenic bacteria and then orally administered alive, as a result, a long-lasting immune response can be elicited. The surface structures of the bacteria which act as adjuvants might explain its strong antigenicity of the surface-expressed foreign protein [45].

In Gram-negative bacteria, heterologous surface display was widely applied and

now become a promising research area. *E. coli* acts a main role as an expression host to display heterologous protein on the cell surface [46, 47]. However, little has been reported on the expression of heterologous surface display on Gram-negative bacteria for the purpose of production of 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 the outer membrane so that the secretion of heterologous proteins becomes simple correspondingly [48]. Therefore, Gram-positive bacteria are considered as good candidates for the development of recombinant live vaccine using surface display expression system. Furthermore, some of Gram-positive bacteria are non-pathogenic or food-grade, which are very desirable to generate live bacterial vaccines for their safety and efficacy. For instance, *Streptococcus gordonii* has been employed to display the tetanus toxin fragment C (TTFC) on the surface of bacteria by Medaglini *et al.* [48]. In addition, *Lactobacillus* has been also 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 [49, 50]. In this study, I am interested to develop a cell surface display system based on *B. subtilis* since it offers several advantages mentioned above. In addition, *B. subtilis* is a bacterium which is closely related to an edible bacterium, *Bacillus subtilis* (natto). Isticato *et al.* [51] have already employed CotB as an anchoring protein to display the tetanus toxin fragment C (TTFC) of *Clostridium tetani* on the surface of *B. subtilis* spores. However, little has been reported whether the heterologous protein can be expressed on the surface of vegetative cells of *B. subtilis*. Therefore, I plan to use *B. subtilis* as a tool for the production of live recombinant bacteria via display of fusion antigen protein on its vegetative cell surface.

To data, four kinds of proteins have been used as cell surface anchoring proteins such as outer membrane protein, lipoproteins, secretory proteins, and surface organ proteins. Ricca *et al.* [52] reported that CotB is one of components of the *B. subtilis* spore coat and has a hydrophilic C-terminal half made up of three repeated 27 amino acid, which is rich in serine, lysine and glutamine residues. Based on analogy to the

connective tissue proteins collagen and elastin, the lysine residues in repeat area were considered to represent the sites of intra- or inter-molecular cross-linking [52, 53]. Therefore, I plan to construct a CotB-based surface display system to express the foreign antigen protein on the cell surface of *B. subtilis* for the production of vaccine delivery vehicle.

As we know, an ovalbumin (OVA) T cell epitope integrating with MHC II class molecule can be recognized by a transgenic mouse having a specific T cell receptor for the ovalbumin epitope so that it can induce OVA-specific T-cell response by oral administration. Therefore, to confirm the antigenicity of displayed antigen protein on the cell surface of *B. subtilis*, OVA T cell epitope was used and linked with CTB which is a good adjuvant and immune carrier to construct fusion antigen. The schematic structure of CotB-based cell surface display system in this study is shown in Figure 1.4. The characterization of the mode of expression of antigen protein on the surface of *B. subtilis* cell is reported in Chapter 3.

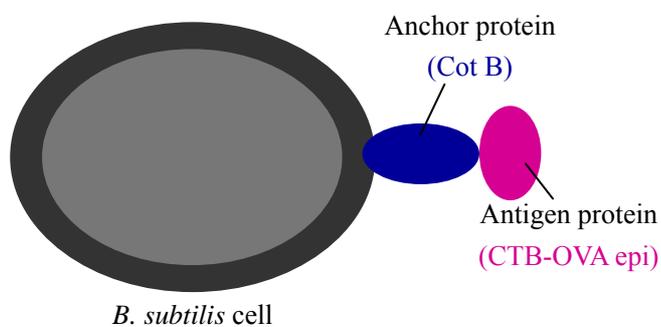


Figure 1.4 Structure of CotB-based cell surface display system in this study

# CHAPTER 2

## Expression of Recombinant Allergens of Japanese Cedar Pollen from a Gene Integrated in the Genome of *Bacillus subtilis*

### 2.1 Introduction

Production of recombinant vaccine in edible bacteria is attractive because of the easy cultivation and rapid harvest compared with plant-derived recombinant vaccine. Genes integrated in the bacterial genome are generally stable, removing the need for harmful antibiotics to maintain the antigen gene. Thus evaluation of expression from genomically-integrated transgenic antigen genes would be very useful for the development of edible vaccines. In this case, *B. subtilis* is a good candidate as an expression organism for the production of vaccine because it offers some advantages over other Gram-positive bacteria as described in Chapter 1. In addition, cholera toxin B subunit (CTB) was used as an adjuvant and antigen-carrier to enhance the mucosal immune response to T cell epitopes of Cry j 1, one of major antigens of Japanese cedar (*Cryptomeria japonica*) pollen [29].

In the previous study, genes encoding epitopes from a major pollen antigen, Cry j 1 or Cry j 2, were fused to CTB gene by stepwise PCR resulting in two fusion genes, *ctb-cry j 1 epi* and *ctb-cry j 2 epi*. The antigenicities of CTB-Cry j 1 epi and CTB-Cry j 2 epi recombinant proteins expressed in *E. coli* were confirmed by western blotting analysis [54]. Furthermore, fusion gene, *ctb-cry j 1 epi*, was integrated into the genome of *B. subtilis* by homologous recombination. The inserted fusion gene was recovered from the genome of *B. subtilis* and then integrated in the pGETS103 plasmid to form pGETS103-ctb-cry j 1 epi recombinant plasmid by homologous recombination. *B. subtilis* 168 was transformed by pGETS103-ctb-cry j 1 epi

recombinant plasmid. CTB-Cry j 1-epi expressed from the pGETS103-ctb-cry j 1 epi recombinant plasmid in *B. subtilis* was confirmed by western blotting analysis. This result indicated that the expression of antigen gene was functional. However, under the same expression condition, the expression of *ctb-cryj1* gene in the genome of *B. subtilis* was not detected by western blotting analysis probably due to low amount of expressed antigen and insufficient sensitivity of the method.

To confirm the expression of antigen protein from the gene in the genome, the use of a detection method with higher sensitivity seems necessary. Therefore, GFP was employed as a reporter of expression in this study. GFP gene was linked to *ctb-cry j 1 epi* fusion gene to construct *ctb-cry j 1-gfp* fusion antigen gene. The *ctb-cry j 1 epi-gfp* fusion gene was then inserted into the genome of *B. subtilis* by homologous recombination [12]. The fluorescence of *B. subtilis* harboring the *ctb-cry j 1 epi-gfp* gene in its genome demonstrated that antigen was successfully expressed. The amount of expressed CTB-Cry j 1 epi-GFP antigen protein from the genome of *B. subtilis* was quantified by western blotting method. I report here the characterization of the mode of expression of fused antigen protein in *B. subtilis*.

## 2.2. Materials and Methods

### 2.2.1 Bacterial strains, plasmids, growth conditions

*B. subtilis* 168 *trpC2*, *B. subtilis* BEST2131, *B. subtilis* BEST20038, pHASH120 vector and pGETS103 vector were kindly gifted by Dr. Mitsuhiro Itaya of the Mitsubishi Kagaku Institute of Life Sciences. The relevant genotypes of these strains were listed in **Table 2.1**. *E. coli* JM109 was used for cloning the recombinant plasmid of pHASH120 (Takara Bio, Shiga, Japan). Ampicillin (50 µg/ml) or chloramphenicol (5 µg/ml) was used as the selection antibiotic for transformed *E. coli* competent cells. **Table 2.2** show the details of plasmids and bacterial strains used in this study. *B. subtilis* and *E. coli* strains were all grown in LB medium at 37 °C. *B. subtilis* transformants were selected with chloramphenicol (5 µg/ml), tetracycline (10 µg/ml)

or blasticidin S (250 µg/ml).

Chloramphenicol, tetracycline, and blasticidin were all purchased from Wako (Osaka, Japan). Ampicillin was purchased from Sigma-Aldrich (Tokyo, Japan).

Table 2.1 *B. subtilis* strains used in this study

Strain	Relevant genotype
<i>B. subtilis</i> 168	Parental strain; <i>trpC2</i>
<i>B. subtilis</i> BEST2131	Parental strain; <i>trpC2 leuB</i> :: pBRTc
<i>B. subtilis</i> BEST20038	<i>leuB</i> ::pHASH102 (BEST2131)

Table 2.2 Plasmids and bacterial strains used in the study

Plasmid	Description	Transformed bacterium strains
pHASH102 (5836bp)	<i>bla cat bsr</i> RBS	<i>B. subtilis</i> BEST20038
pHASH120 (6026bp)	<i>bla cat bsr</i> PS10-RBS	<i>E. coli</i> JM109 <i>B. subtilis</i> BEST2131
pHASH120-ctb-cry j1 epi-gfp (7259bp)	<i>bla cat bsr</i> PS10-RBS- <i>ctb-cry j1 epi-gfp</i>	<i>E. coli</i> JM109 <i>B. subtilis</i> BEST2131
pGETS103 (14.5kb)	<i>bla tet tetL</i>	<i>E. coli</i> JM109
pGETS103-ctb-cry j1 epi-gfp (15.9kb)	<i>cat tetL</i> PS10-RBS- <i>ctb-cry j1 epi-gfp</i>	<i>B. subtilis</i> 168 <i>trpC2</i>

\*Antibiotic-resistance genes indicated are: *bla*, ampicillin; *cat*, chloramphenicol; *bsr*, blasticidin S; *tet*, tetracycline (for *E. coli*); *tetL*, tetracycline (for *B. subtilis*).

### 2.2.2 Construction of fusion antigen genes

In former experiments, genes encoding epitopes from Cry j 1 or Cry j 2 were linked and then fused to CTB gene by stepwise PCR to construct two fusion genes, *ctb-cry j 1 epi* and *ctb-cry j 2 epi*. The antigenicities of CTB-Cry j 1 epi and CTB-Cry j 2 epi

proteins expressed in *E. coli* were confirmed by corresponding antibody using western blotting analysis.

To examine the mode of expression of antigen protein from the gene in the genome of *B. subtilis*, the *ctb-cry j 1 epi* fusion gene was linked to *gfp* by PCR. The C-terminus of the CTB-Cry j 1 epi antigen peptide was fused to the GFP peptide sequence via a GGSSGA linker [55]. The *gfp* gene sequence was amplified from the pEGFP plasmid (BD Biosciences, Tokyo, Japan) using the forward primer (5'-ATGGTGAGCAAGGGCGAGGAGCTGTTCACC-3') and the reverse primer (5'-TTACTTGTACAGCTCGTCCAT-3'). The sequence of the linker was attached to the *ctb-cry j 1 epi* gene by PCR using the forward primer (5'-ACACCTCAAAATATTACTG-3') complementary to the 5' end of the *ctb* gene and the reverse primer (5'-*TCGCCCTTGCTCACCATTGCTCCGCTGCTTCCTCCCGGGCCGA*ACTGGTT3') in which the sequence indicated in italics is complementary to *gfp*, the sequence indicated in bold is the linker, and the sequence underlined is complementary to the *ctb-cry j 1 epi* gene). KOD DNA polymerase (Toyobo, Osaka, Japan) was used for these PCR reactions. Finally, *ctb-cry j 1 epi-gfp* was constructed using an overlap extension PCR method. The two PCR products, *ctb-cry j 1 epi-linker-gfp* (containing 17 bp of the 5' end sequence of *gfp*) and *gfp*, were mixed, and 10 PCR cycles were performed with denaturation at 90 °C, annealing at 63 °C and extension by KOD polymerase at 68 °C. Then, the forward primer (5'-ATGACACCTCAAAATATTACTG-3') complementary to the 5' end sequence of *ctb*, and the reverse primer (5'-TTACTTGTACAGCTCGTCCAT-3') complementary to the 3' end sequence of *gfp* were added to amplify the final PCR product *ctb-cry j 1 epi-gfp*. Taq polymerase (Takara Bio, Otsu, Japan) was used for this PCR reaction to add a dA nucleotide onto both ends of the PCR product to facilitate TA cloning. All the primers were purchased from Invitrogen Japan (Tokyo, Japan). The structure of the fusion gene is shown in **Figure 2.1**. DNA sequences for the insertion into plasmid were checked using the dye-terminator method of Bio Matrix Research Institute (Nagareyama, Japan).

All the primers were purchased from Invitrogen Japan (Tokyo, Japan).

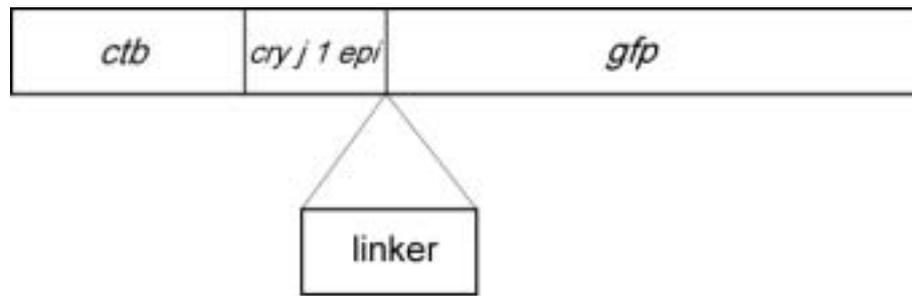


Figure 2.1 Structure of the fusion antigen gene for integration into the genome of *B. subtilis*. The fusion antigen gene (*ctb-cry j 1 epi-gfp*) was constructed from the cholera toxin B subunit gene (*ctb*, 312 bp including the initiation codon), five T-cell epitopes from the Cry j 1 antigen (*cry j 1 epi*, 189 bp), the linker sequence (GGSSGA, 18 bp), and the green fluorescent protein gene (*gfp*, 720 bp including the termination codon).

### 2.2.3 Construction of recombinant plasmid

#### 2.2.3.1 Introduction of pHASH120 vector

A novel cloning vector, named as pHASH120, was constructed by Ohashi *et al.* [12]. **Figure 2.2** shows the structure of pHASH120 vector. Upstream of RBS, *Sma*I is positioned for the cloning of optional promoters using the T-A cloning method. PS10 was employed as the promoter because it is one of the strongest promoters in *B. subtilis*. The consensus RBS determined by searching the whole genome sequence of *B. subtilis* was AAAGGAGG [12]. This corresponded to the experimental data showing the optimal RBS as (A/T) AAGGAGG and a 7 to 9-nucleotide-spacing between RBS and start codon [12]. An interested gene amplified by PCR can be cloned in the T-extended *Eco*RV ends by T-A cloning method. Plasmid pHASH120 contains the ampicillin-resistant gene and the part of tetracycline-resistant ( $\Delta$ tet) genes which are all derived from pBR322 vector, the sequence between them can be integrated in the genome of bacteria by homologous recombination.

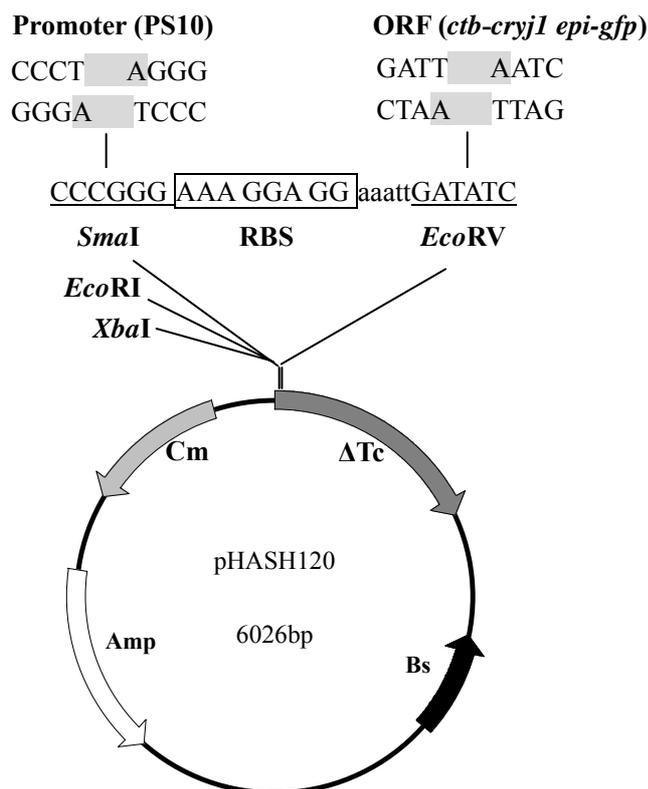


Figure 2.2 Schematic structure of pHASH120 vector and the insertion site of the antigen gene. Antibiotic-resistance genes indicated are: Amp, ampicillin; Cm, chloramphenicol;  $\Delta$ Tc, part of tetracycline-resistant gene; Bs, blasticidin S

### 2.2.3.2 Preparation of pHASH120 T vector

T vector was prepared according to the method described by Ohashi *et al.* [12]. A 2-  $\mu$ g amount of pHASH120 was digested completely with 30 U of *EcoRV* at 37 °C for 6 h and treated with phenol/chloroform extraction. After precipitation with ethanol, DNA was dissolved in 10  $\mu$ l of TE buffer. An aliquot of 5  $\mu$ l of the digested plasmid solution was treated with *Ex Taq* DNA polymerase (Takara Bio, Ohtsu, Japan) at a ratio of 2.5 U/ $\mu$ g plasmid in 50  $\mu$ l buffer solution (1  $\times$  *Ex Taq* buffer supplemented with 2mM dTTP) at 72 °C for 2 h. After phenol/chloroform extraction twice and isopropanol precipitation, the T-vector was prepared by dissolving in 10  $\mu$ l of TE buffer and stored at -80 °C until use.

### **2.2.3.3 Transformation of *E. coli* by pHASH120-ctb-cry j 1 epi-gfp recombinant plasmid**

The antigen gene *ctb-cry j1 epi-gfp* amplified by PCR was introduced into the T-vectors at 16 °C overnight using DNA Ligation Kit (Takara Bio, Otsu, Japan) as described in the manual. The recombinant plasmid pHASH120-ctb-cry j1 epi-gfp was amplified in *E. coli* JM109 described below. Solution after plasmid ligation (10 µl) was incubated with *E. coli* JM109 competent cells (100 µl) for 30 min on ice, followed by incubation at 42 °C for 45sec. After 2 min on ice, 1ml SOC medium was added. Solution was grown at 37 °C under shaking at 200 rpm for 1 h. Then, 150 µl solution was spread on LB plate supplemented with an appropriate antibiotic, followed by incubation at 37 °C overnight. Since there are two possible orientations of *ctb-cry j1 epi-gfp* inserted in the T-extended *EcoRV* ends of pHASH120 using T-A cloning method, the forward primer *EcoRI-SmaI-RBS-FW* (5'GCGACGGAAATGT TGAATACTCAT3') and the reverse primer *gfp-RV* (5'TTACTTGACAGCTCGTC CAT3') were used to confirm the desired orientation of inserted gene in the recombinant plasmid by colony PCR.

### **2.2.3.4 Extraction of pHASH120-ctb-cry j 1 epi-gfp recombinant plasmid**

Recombinant plasmid pHASH120-ctb-cry j 1 epi-gfp from *E. coli* JM109 was extracted using Spin MiDiprep Kit (Qiagen, Tokyo, Japan) as described in the manual. The sequence of *ctb-cry j1 epi-gfp* fusion gene inserted in pHASH120 plasmid was confirmed by Biomatrix company.

## **2.2.4 Integration of fusion antigen gene in the genome of *B. subtilis***

### **2.2.4.1 The principle of integration of fusion antigen gene in *B. subtilis* genome**

*B. subtilis* BEST2131 was constructed from 168 *trpC2* strain. It has pBR322 sequences and an insertion of tetracycline-resistant gene between them in the *leuB* gene (*leuB::pBRTc*) of *B. subtilis* 168 *trpC2* genome. *B. subtilis* BEST2131 genome and pHASH120-ctb-cry j 1 epi-gfp recombinant plasmid all contain the

ampicillin-resistant and the part of tetracycline-resistant ( *tet*) genes derived from pBR322 vector, the sequence between them can exchange naturally. This process is defined as homologous recombination. As a result, *B. subtilis* BEST2131 genome obtains the *ctb-cry j1 epi-gfp* antigen gene together with the promoter and RBS, which function to express antigen peptide in *B. subtilis* genome. *B. subtilis* BEST2131 with *ctb-cry j1 epi-gfp* inserted into the genome becomes resistant to chloramphenicol and sensitive to tetracycline and blasticidin S. **Figure 2.3** shows the schematic insertion process of the antigen gene into the genome of BEST2131 and the antibiotic resistance before and after the insertion of the antigen gene.

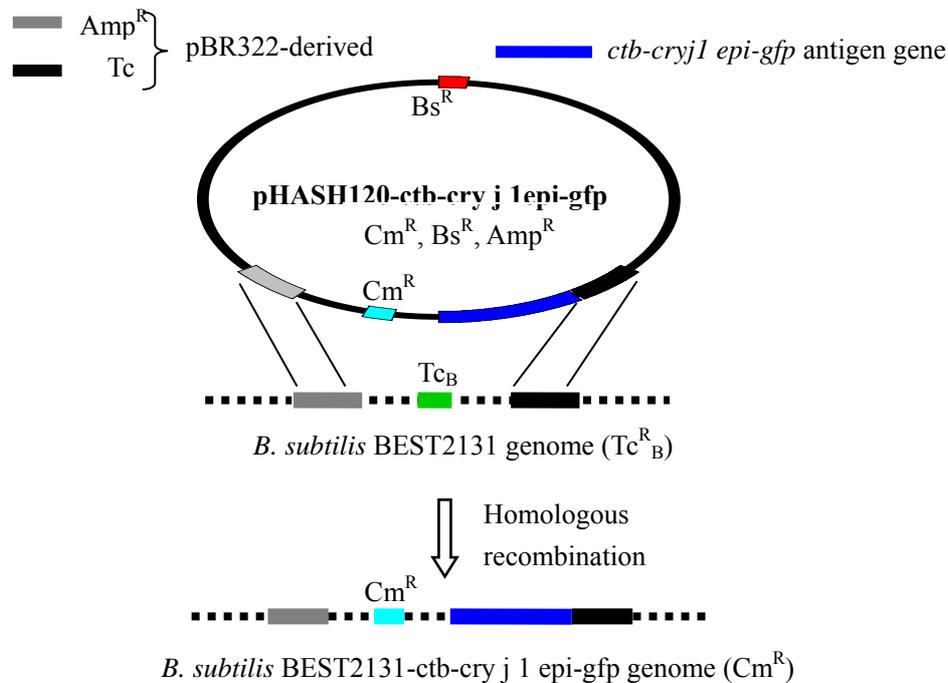


Figure 2.3 Procedure of integration antigen gene into *B. subtilis* BEST2131 genome by homologous recombination. Antibiotic-resistance genes indicated are: Amp, ampicillin; Cm, chloramphenicol;  $\Delta$ Tc, part of tetracycline-resistant gene; Bs, blasticidin S; *Tc<sub>B</sub><sup>R</sup>*, tetracycline-resistant gene only functional in *B. subtilis*.

#### 2.2.4.2 Transformations of *B. subtilis* BEST2131 by pHASH120-*ctb-cry j1 epi-gfp* recombinant plasmid

Transformations of *B. subtilis* BEST2131 were performed according to the method

described below [56]. Solutions used for the transformation of *B. subtilis* were shown in **Table 2.3** and **Table 2.4**.

Table 2.3 Solutions used for the transformation of *B. subtilis*

Component	10×Spizizen (ml)	Medium A (ml)	Medium B (ml)
K <sub>2</sub> HPO <sub>4</sub>	140g	14g	
KH <sub>2</sub> PO <sub>4</sub>	60g	6g	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20g		2g
C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> Na <sub>3</sub> · 2H <sub>2</sub> O	10g		1g
H <sub>2</sub> O	to 1000	to 50	to 50

Table 2.4 Solutions used for the transformation of *B. subtilis*

Component	TF-1 (ml)	TF-II (ml)	TF-D (ml)
10×Spizizen soluton	100	100	
Medium A			0.625
Medium B			6.25
50 % glucose	10	10	1.25
2 % MgSO <sub>4</sub> · 7H <sub>2</sub> O	10	10	
2 % casamino acid	10	5	
5 mg/ml tryptophan	10	1	1.25
5 mg/ml arginine	10	1	1.25
5 mg/ml leucine	10	1	
5 mg/ml histidine			1.25
5 mg/ml threonine	10	1	
H <sub>2</sub> O	to 1000	to 1000	to 100

A single colony of *B. subtilis* strain BEST2131 was inoculated in 1ml LB medium and grown at 37 °C for 12 h under shaking (200 rpm). Fifty µl of culture solution was

mixed with 50  $\mu$ l of 2 % casamino acid and 1ml of TF-I, and then cultured at 37 °C for 4 h under shaking. Then, 0.4 ml of the TF-I culture was mixed with 3.6 ml of TF-II and cultured at 37 °C for 1 h under shaking (200 rpm). After the centrifugation at 4000rpm for 15min at 4 °C, 175  $\mu$ l of TF-II and 75  $\mu$ l of 50 %glycerol were added to suspend the cell pellet to prepare competent cells. Competent cells (6.25  $\mu$ l), 1.25  $\mu$ l of 2 %  $MgSO_4 \cdot 7H_2O$ , 1.25 $\mu$ l of 1M  $MgCl_2$ , and 5  $\mu$ l of pHASH120-ctb-cry j 1 epi-gfp vector used to transform *B. subtilis* BEST2131 were added in 50  $\mu$ l TF-D solution. The solution was incubated at 37 °C for 1 h, followed by the addition of 200  $\mu$ l of LB medium and 1 h incubation at 37 °C under shaking (200 rpm).

Transformants (BEST2131 transformed by pHASH120-ctb-cry j1 epi-gfp vector) were selected on LB plate containing tetracycline (10  $\mu$ g/ml), blasticidin S (250  $\mu$ g/ml), or chloramphenicol (5  $\mu$ g/ml) by incubation at 37 °C overnight respectively. Colonies which can grow on LB plate containing chloramphenicol were chosen as positive transformants (named *B. subtilis* BEST2131-ctb-cry j 1 epi-gfp).

#### **2.2.4.3 Confirmation of antigen gene inserted in *B. subtilis* genome**

The genomes were extracted using QIAamp DNA mini Kit (Qiagen , Tokyo, Japan). The insertion of *ctb-cry j1 epi-gfp* in the genome of *B. subtilis* was confirmed by PCR using CTB-FW primer and GFP-RV primer.

#### **2.2.5 Recovery of fusion antigen gene from the genome of *B. subtilis* to plasmid**

##### **2.2.5.1 Introduction of pGETS103 vector**

The structure of pGETS103 is shown in **Figure 2.4**. The vector of pGETS103 consists of *B. subtilis* plasmid pTB522 and the full length of *E. coli* plasmid pBR322, and can shuttle between *E. coli* and *B. subtilis*. A multicopy plasmid, pTB522, from a thermophilic *Bacillus* strain can grow by  $\theta$ -type replication in *B. subtilis*. The  $\theta$ -type replication was demonstrated to carry large DNAs stably compared with the rolling circle replicating form common to many plasmids in Gram-positive bacteria. The vector of pGETS103 has two antibiotic resistance genes from pBR322 (ampicillin

resistance and tetracycline resistance, which are only functional in *E. coli*), and one from pTB522 (tetracycline resistance, which is only functional in *B. subtilis*). Between pBR322-derived antibiotic resistance genes, there is a *Hind* III site, which was used for the linearization of the vector.

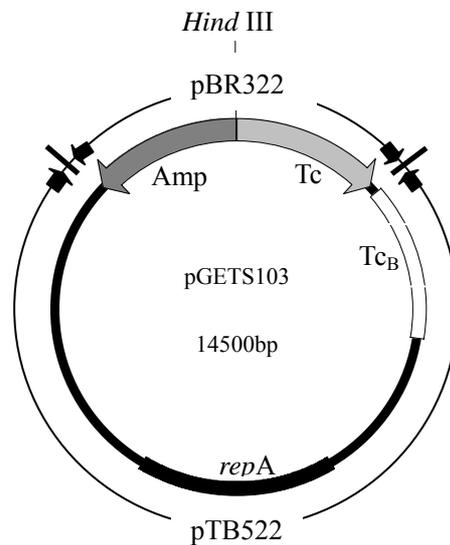


Figure 2.4 Schematic structure of pGETS103 vector. Antibiotic-resistance genes indicated are: Amp, ampicillin; Tc, tetracycline-resistant gene, only functional in *E. coli*; Tc<sub>B</sub>, tetracycline-resistant gene, only functional in *B. subtilis*.

The copy numbers of pGETS103 were estimated to be about 9 for *B. subtilis* and about 20 for *E. coli* [57]. The vector of pGETS103 was linearized before transformation. The linearized pGETS103 is unable to self-circularize, and circular pGETS103 can be established only after recovering the antigen gene from the genome by the homologous recombination.

#### 2.2.5.2 The principle of recovery of fusion antigen gene from *B. subtilis* genome

Linearized pGETS103 was prepared by digesting 1 µg of pGETS103 with 10 U of *Hind* III at 37 °C for 2 h, followed by phenol/chloroform extraction and ethanol precipitation. The linearized pGETS103 was used to transform *B. subtilis* BEST2131-ctb-cry j 1 epi-gfp to recover the antigen gene from the genome by homologous recombination. **Figure 2.5** shows the schematic insertion process of

recovery antigen gene from the genome of *B. subtilis* BEST2131 to the pGETS103 vector and the change of antibiotic resistance.

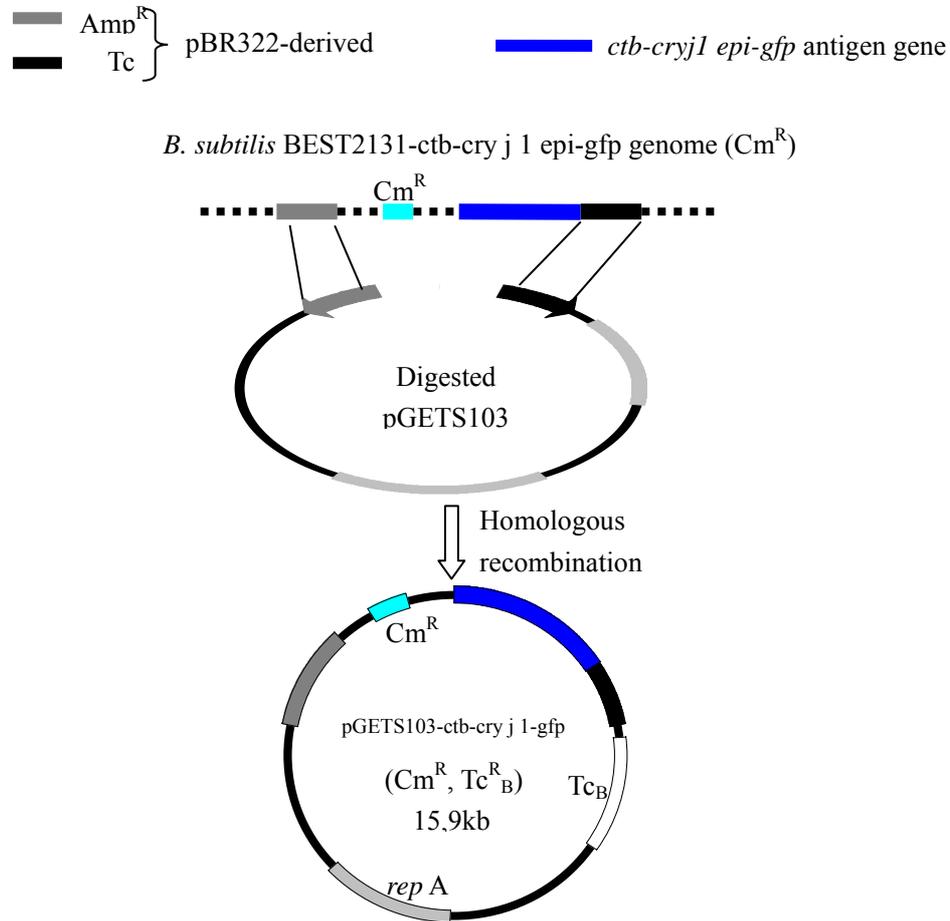


Figure 2.5 Schematic procedure of recovery antigen gene from the genome of *B. subtilis* BEST2131 to the plasmid

### 2.2.5.3 Transformation of *B. subtilis* BEST2131-*ctb-cry j 1-epi-gfp* with linearized pGETS103

Transformation of *B. subtilis* BEST2131-*ctb-cry j 1-epi-gfp* with linearized pGETS103 was performed by the method described in Materials and Methods 2.2.4.2. A single colony of *B. subtilis* BEST2131-*ctb-cry j 1-epi-gfp* instead of *B. subtilis* BEST2131 was inoculated in 1 ml LB medium and grown at 37 °C for 12 h under shaking (200 rpm). Five µl of linearized pGETS103 instead of pHASH120-*ctb-cry j 1 epi-gfp* vector was mixed with other solution for the final transformation.

Transformants (*B. subtilis* BEST2131-ctb-cry j1 epi-gfp transformed by pGETS103 digested by Hind III) were selected on LB plate containing tetracycline (10 µg/ml) or chloramphenicol (5 µg/ml) by incubation at 37 °C overnight respectively. Colonies which only can grow on LB plate containing chloramphenicol were chosen as positive transformants. The recombinant plasmid of pGETS103 containing the antigen gene was recovered from the positive transformant.

#### **2.2.5.4 Extraction of pGETS103-ctb-cry j 1 epi-gfp recombinant plasmid**

Highspeed Plasmid Midi Kit (Qiagen, Tokyo, Japan) was used to extract of pGETS103-ctb-cry j1 epi-gfp from *B. subtilis* BEST2131 using the method described below. *B. subtilis* cells were pelleted from 200 ml of LB culture medium (OD<sub>600</sub>=1.2) under centrifugation at 3,000×g for 15min at 4 °C and resuspended in 8 ml of Buffer P1 containing 5 mg/ml lysozyme (Wako, Osaka, Japan) and incubated at 37 °C for 30 min. Eight ml of Buffer P2 was added and mixed gently but thoroughly by inverting 4-6 times, and then incubated at room temperature for 5 min. Pre-chilled 8 ml of Buffer P3 was added and mixed, then incubated on ice for 15 min followed by centrifugation at 20,000×g for 30 min at 4 °C twice. The supernatant was added into QIAGEN-tip equilibrated with 4 ml of Buffer QBT, and allowed the column to empty by gravity flow. Then QIAGEN-tip was washed with 10 ml of Buffer QC twice, and allowed the column to empty by gravity flow. Plasmid eluted with 5 ml of Buffer QF was precipitated by adding 3.5 ml of isopropanol at room temperature and centrifuging a 15,000×g for 30 min at 4 °C, followed by washing with 2 ml of 70 % ethanol and centrifuged at 15,000×g for 10 min at 4 °C. Finally, the pellet was air-dried and redissolved in 50 µl of the elution buffer TE.

Primers of CTB-FW and GFP-RV were used to confirm the recovery of *ctb-cry j1 epi-gfp* in the recombinant plasmid by PCR method.

#### **2.2.5.5 Transformations of *B. subtilis* 168 *trpC2* with pGETS103-ctb-cry j 1 epi-gfp recombinant plasmid**

Transformation of *B. subtilis* 168 *trpC2* was performed using the electroporation

method described below [58]. *B. subtilis* 168 *trpC2* competent cells for electroporation were prepared firstly. A single colony of *B. subtilis* 168 *trpC2* was firstly incubated in 2 ml of LB medium at 37 °C for 12 h under shaking (200 rpm). Then, all culture solution was added in 200 ml of LB medium and incubated at 37 °C under shaking (250 rpm) until OD<sub>600</sub> reached 1.5-2.0. The cells were harvested by centrifugation at 3,000×g for 10 min and washed three times with 200ml sterile cold ultrapure water and resuspended in 2 ml of pre-chilled 30 % polyethyleneglycol (PEG) 6000. The cells were then dispensed into 100 µl aliquots, rapidly frozen and stored at -80 °C.

The recombinant plasmid, pGETS103-ctb-cry j1 epi-gfp, was dialysed for 1h using the semipermeable membrane MWCO 6-8000 against deionized water under stirring and concentrated to the original volume by centrifugal filter devices Microcon YM-10.

Electroporation was performed using a BTX electroporation system ECM-600. The prepared *B. subtilis* 168 competent cells (100 µl) were firstly thawed on ice. The 10 µl of dialyzed vector was added in the cells with gentle mixture and then transferred to a pre-chilled electroporation cuvette (2mm gap). This cuvette was placed on ice for 10min before electroporation. Electroporation was performed at 2.5V, with the pulse controller set at 186ohms. Then, the cells were added in 2 ml of SOC medium and incubated at 37 °C under gentle shaking (160rpm) for 1.5 h to allow expression of the antibiotic resistance. Chloramphenicol (0.2 µg/ml) was added to the transformed cells in SOC after 1 h incubation to induce resistance. Cells were then concentrated to 0.1 ml and plated on LB plate containing chloramphenicol (5 µg/ml).

## **2.2.6 Expression of antigen protein in *B. subtilis***

### **2.2.6.1 Fluorometric measurement**

Spizizen's minimal medium (SMM) plate supplemented with 0.01 % L-tryptophan, 0.01 % yeast extract, 0.2 % casamino acids, and 1.5 % agar were used for visualization of colony fluorescence of *B. subtilis* strains [59, 60]. The colonies of *B.*

*subtilis* grown on this culture plate were cultivated at 37 °C for 2 days. Fluorescence from *B. subtilis* excited under blue LED light at 470 nm was monitored on a luminescent image analyzer LAS 1000 CH equipped with a highly sensitive CCD camera (Fuji photo film, Tokyo, Japan).

A single positive colony of *B. subtilis* 20038 and *B. subtilis* BEST2131 transformed by pHASH120-ctb-cry j1 epi-gfp was precultured in 2 ml of LB medium containing 5 µg/ml of chloramphenicol at 37 °C for 12 h under shaking (200rpm). Then, 50 µl each of preculture was added in six tubes containing 5ml LB medium and 5 µg/ml of chloramphenicol and incubated at 37 °C for 6 h, 8 h, 10 h, 12 h, 14 h, and 16 h under shaking (250 rpm). The turbidity at 660 nm of samples was monitored by a UV spectrophotometer mini 1240 (Shimadzu, Japan). An equal number of cells was collected by centrifugation at 3,500×g for 5 min at 4 °C. Cells were washed twice with 1ml of water followed by resuspension in water (OD<sub>660</sub>=1). Fluorescence intensities of *B. subtilis* were measured by a fluorescence spectrophotometer F2500 (Hitachi High-Technologies, Tokyo, Japan) with 1 cm optical path length using excitation at 395 nm and emission at 509 nm.

#### **2.2.6.2 Time course of expressed antigen protein from the gene in the genome of *B. subtilis***

To confirm the expression of antigen protein from the gene in the genome of *B. subtilis*, *B. subtilis* BEST2131 transformed by pHASH120-ctb-cry j1 epi-gfp was cultured as described in 2.2.6.1. The bacterial culture was diluted to OD<sub>660</sub> = 1.0, and then 10 ml of cells were pelleted by centrifugation at 15,000 rpm for 5 min at 4 °C and resuspended in 50 µl of 2 × SDS-PAGE sample buffer. The suspensions were then heated for 5 min at 90 °C. This operation was repeated three times in order to improve the yield of protein. Then the suspensions were centrifuged at 15,000 rpm for 5 min at 4 °C. Fifteen µl of supernatant were used for the SDS-PAGE, followed by western blotting analysis.

### **2.2.6.3 Comparison of the expression of antigen protein from the gene in the genome and from the plasmid in *B. subtilis***

To quantify the expressed antigen protein from the gene in the genome of *B. subtilis* and from the plasmid in *B. subtilis*, a single colony of *B. subtilis* 20038 (negative control), *B. subtilis* BEST2131 transformed by pHASH120-ctb-cry j1 epi-gfp, and *B. subtilis* 168 *trpC2* transformed by pGETS103-ctb-cry j1 epi-gfp were precultured in 2 ml of LB medium containing 5 µg/ml of chloramphenicol at 37 °C for 12 h under shaking respectively. Then, 50 µl of preculture were added into 5ml of LB medium containing 5 µg/ml of chloramphenicol and incubated at 37 °C for 10 h. Bacteria were pelleted by centrifugation and 50 µl of 2×SDS-PAGE sample buffer was added. The extraction of proteins was performed by heating the suspensions for 5 min at 90 °C. This operation was also repeated three times in order to improve the yield of protein. Then the suspensions were centrifuged at 15,000 rpm for 5 min at 4 °C. Fifteen µl of supernatant were used for the SDS-PAGE, followed by western blotting analysis.

Total protein expressed in *B. subtilis* was measured with BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Tokyo, Japan). BandsScan version 4.30 was used to analyze the amount of expressed antigen protein in *B. subtilis*.

### **2.2.6.4 Western blotting**

Proteins expressed in *B. subtilis* were separated on SDS-PAGE gels and then transferred onto PVDF transfer membrane (Amersham Bioscience, Tokyo, Japan) using the semi-dry method [61]. Rabbit anti-cholera toxin antibody (C3062) (Sigma-Aldrich Japan, Tokyo, Japan) or rabbit anti-GFP antibody (A6455) (Invitrogen Japan, Tokyo, Japan) was used as the primary antibody for CTB-Cry j 1 epi-GFP antigen protein. The secondary antibody was donkey anti-rabbit antibody conjugated with horseradish peroxidase (NA 934V) (Amersham Bioscience, Tokyo, Japan). The signal was detected with enhanced chemiluminescence reagents (Amersham Bioscience, Tokyo, Japan). CTB (Sigma-Aldrich Japan, Tokyo, Japan) and GFP (Sigma-Aldrich Japan, Tokyo, Japan) were used as the positive controls.

## 2.3. Results

### 2.3.1. Construction of *ctb-cry j 1-gfp* fusion antigen gene

To examine the mode of expression of antigen protein from the genomically-integrated transgene, the *ctb-cry j 1 epi* fusion gene was linked to GFP gene by PCR, to generate a *ctb-cry j 1 epi-gfp* fusion gene (**Figure 2.1**). Electrophoretic analysis of PCR product was shown in **Figure 2.6**. Results of sequencing analysis showed that DNA and deduced amino acid sequences were identical to the designed ones. The sequences of nucleotide and amino acid of CTB-Cry j1 epi-GFP antigen protein were shown in **Figure 2.7**. The nucleotide sequence of the *ctb-cry j 1 epi-gfp* gene has been registered in DDBJ (accession number AB480283).

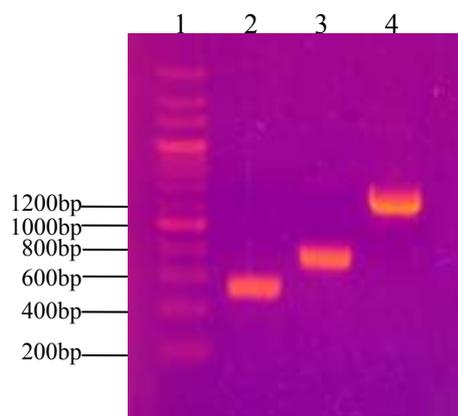


Figure 2.6 Electrophoretic analysis of PCR product. Lane 1, 200bp DNA ladder; Lane 2, *ctb-cry j 1-epi-linker* fusion gene (516bp); Lane 3, *gfp* gene; Lane 4, *ctb-cry j 1-epi-gfp* fusion antigen gene.

Amino acid sequence of CTB-Cry j1 epi-GFP fusion antigen

TPQNITDLCAEYHNTQIH TLNDKIFSYTESLAGKREMAITFKNGATFQVEVPG  
SQHIDSQKKAIERMKD TLR IAYL TEAKVEKLCVWNNKTPHAIAAISMANQNR  
MKLADCAVGF GSKMPMYIAGYKTFDGRPCVFIKRVSNVIIHGLHLYGSMKVTVAF  
NQFGP**GGSSG**AMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYG

KLTLKFICTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKQHDFFKSAMPEGY  
VQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNY  
NSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPD  
NHYLSTQSALS KDPNEKRDHMLLEFVTAAGITLGMDELYK (411a.a.)

DNA sequence of CTB-Cry j1 epi-GFP fusion antigen

ACACCTCAAATATTACTGATTTGTGTGCAGAATACCACAACACACAAATA  
CATACGCTAAATGATAAGATATTTTCGTATACAGAATCTCTAGCTGGAAAAA  
GAGAGATGGCTATCATTACTTTTAAGAATGGTGCAACTTTTCAAGTAGAAGT  
ACCAGGTAGTCAACATATAGATTCACAAAAAAAAGCGATTGAAAGGATGA  
AGGATACCCTGAGGATTGCATATCTTACTGAAGCTAAAGTCGAAAAGTTATG  
TGTATGGAATAATAAAACGCCTCATGCGATTGCCGCAATTAGTATGGCAAAT  
CAGAACCGTATGAACTGGCGGATTGCGCGGTGGGCTTCGGCAGCAAATGCC  
GATGTATATTGCGGGCTATAAAACGTTTCGATGGCCGTCCGTGCGTGTTCAATAA  
CGTGTGAGCAACGTGATTATTCATGGCCTGCATCTGTATGGCAGCATGAAAGTG  
ACGGTGGCGTTCAACCAGTTCGGCCCGGGAGGAAGCAGCGGAGCAATGGTG  
AGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCT  
GGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG  
GGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGC  
AAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTG  
CAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAG  
TCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC  
GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCT  
GGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACA  
TCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAACGTCTATATCA  
TGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCAC  
AACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACAC  
CCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCA  
CCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC  
CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCT

GTACAAG (1233bp)

Figure 2.7 Sequences of nucleotide and amino acid of CTB-Cry j1 epi-GFP fusion antigen. The sequence underlined indicated CTB (309 bp, 103 a.a); the sequence in italic indicated Cry j 1 epi (189 bp, 63 a.a); the sequence in red indicated linker (18 bp, 6 a.a); and the sequence without emphasis indicated GFP (717bp, 239 a.a).

### 2.3.2. Expression of CTB-Cry j 1-GFP antigen from the gene in the genome of *B. subtilis*

#### 2.3.2.1 Fluorescence characteristics of *B. subtilis* strains containing *ctb-cry j 1 epi-gfp* gene

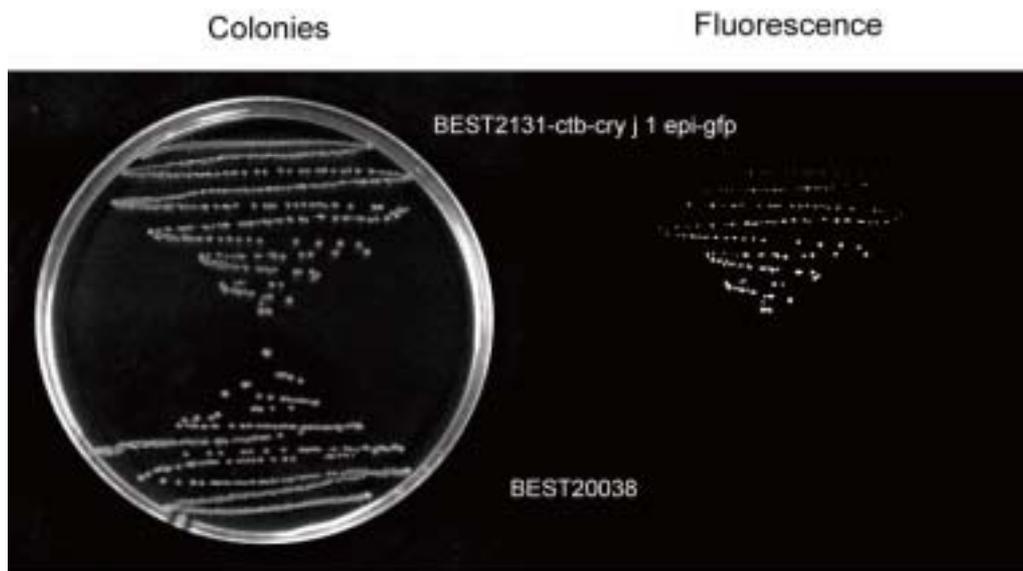


Figure 2.8 Fluorescence characteristics of *B. subtilis* strains. Colonies of *B. subtilis* BEST2131-ctb-cry j 1-gfp (upper half) and *B. subtilis* BEST20038 (negative control, lower half) grown on the plate were photographed under white light (left) or blue LED light at 470nm (right).

To examine the mode of expression of antigen protein from the gene in the genome of *B. subtilis*, the *ctb-cry j 1 epi* fusion gene was further linked with the green fluorescent protein (GFP) gene by PCR method to generate *ctb-cry j 1-gfp* gene. The

*ctb-cry j 1-gfp* fusion antigen gene was firstly cloned into pHASH120 vector by T-A cloning method and then inserted in the genome of *B. subtilis* BEST2131 by homologous recombination. The expression of antigen protein from the gene in the genome of *B. subtilis* was confirmed according to detect the fluorescence characteristics of antigen protein. *B. subtilis* BEST2131-*ctb-cry j 1-gfp* containing *ctb-cry j 1-gfp* gene in its genome was grown on a culture plate at 37°C for 2 days. Fluorescence from the colonies was measured by a CCD camera under blue LED light at 470nm. *B. subtilis* BEST2131-*ctb-cry j 1-gfp* showed green fluorescence but *B. subtilis* BEST20038 (negative control) showed no fluorescence (**Figure 2.8**). *B. subtilis* BEST20038 has the same chromosome structure with *B. subtilis* BEST2131 except that it does not contain an inserted gene and a promoter [12].

### **2.3.2.2 Fluorescence intensities of *B. subtilis* strains containing *ctb-cry j 1 epi-gfp* gene**

The growth curve of *B. subtilis* BEST2131-*ctb-cry j 1-gfp* was monitored by the turbidity at 660 nm with intervals of 2 h. Under the same conditions, fluorescence intensities at 509 nm (bacterial concentration,  $OD_{660}=1.0$ ) of *B. subtilis* BEST2131-*ctb-cry j 1-gfp* and *B. subtilis* BEST20038 (negative control) were monitored by a fluorescence spectrophotometer. *B. subtilis* BEST2131-*ctb-cry j 1 epi-gfp* evidently showed an emission peak at 509 nm which is in accordance with the emission peak of GFP. *B. subtilis* BEST20038 did not show a peak at 509 nm (**Figure 2.9**). **Figure 2.10** shows that cells reached the early stationary growth phase in 10 h. A maximal fluorescence intensity from *B. subtilis* BEST2131-*ctb-cry j 1-gfp* was observed at 10 h and the fluorescence intensity decreased gradually after 10 h (**Figure 2.10**). Almost no change of the fluorescence intensities of *B. subtilis* BEST20038 was observed during the culture (data not shown).

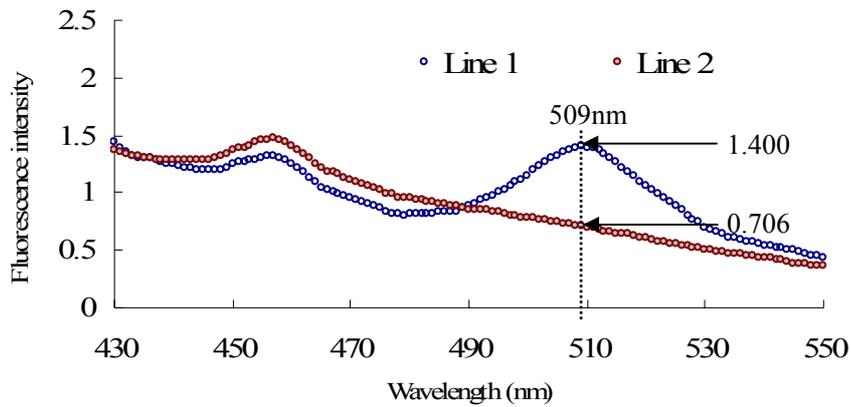


Figure 2.9 Fluorescence intensities of *B. subtilis* strains. Line 1 indicates the fluorescence intensity of *B. subtilis* BEST2131-ctb-cry j 1-gfp at 509 nm ( $OD_{660}=1.0$ ); Line 2 indicates the fluorescence intensity of *B. subtilis* BEST20038 (negative control) at 509 nm ( $OD_{660}=1.0$ ).

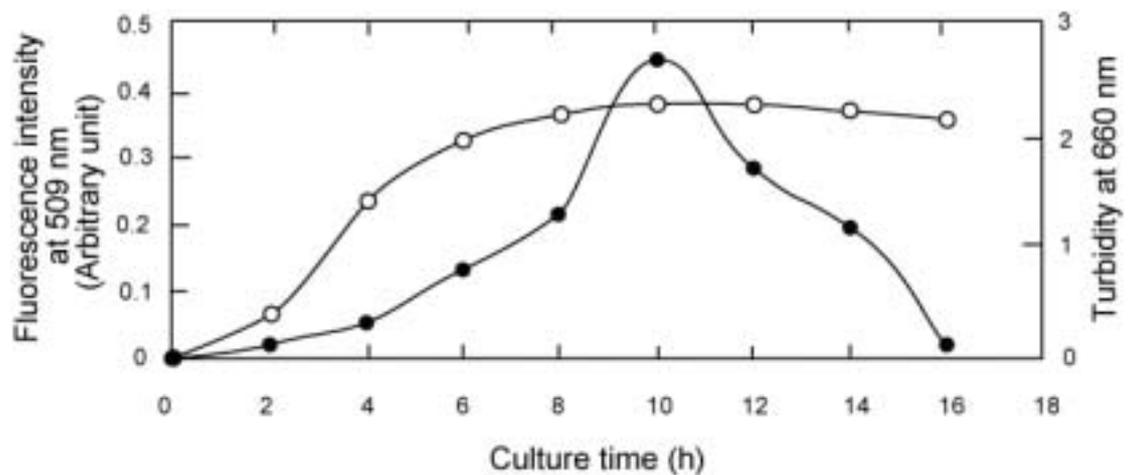


Figure 2.10 Time course of the bacterial growth and fluorescence intensity of *B. subtilis* strains. Closed circles indicate the peak height of fluorescence intensity of *B. subtilis* BEST2131-ctb-cry j 1-gfp at 509 nm ( $OD_{660}=1.0$ ); Open circles indicate the growth curve of *B. subtilis* BEST2131-ctb-cry j 1-gfp ( $OD_{660}$ ). The peak height at 509 nm was measured and background fluorescence was subtracted.

### 2.3.2.3 Confirmation of optimal expression condition of CTB-Cry j 1 epi-GFP antigen protein

Under the same conditions, western blotting was used to identify the optimal expression time of the CTB-Cry j 1 epi-GFP antigen protein. **Figure 2.11** shows that a maximal amount of expressed protein corresponding to the theoretical molecular weight of CTB-Cry j 1 epi-GFP (46 kDa) was observed at 10 h (lane 5, **Figure 2.11(a)**), which concurs with the maximum fluorescence at 10 h. The expressed antigen proteins became degraded with increasing incubation time after 10 h (**Figure 2.11 (a)**). This is probably due to proteases produced in *B. subtilis*, because smaller proteins (29 and 25 kDa), assumed to be proteolytic products, appeared after 8 h (lanes 4 to 8, **Figure 2.11 (a)**). I postulated that the 29 kDa protein was a proteolyzed fragment containing GFP released from the CTB-Cry j 1 epi-GFP antigen protein. Accordingly, no signal was observed at 29 kDa when anti-cholera toxin antibody was used as the primary antibody (**Figure 2.11 (b)**). This result indicates that the protein of about 29kDa is the degraded fragment containing GFP or a part of GFP.

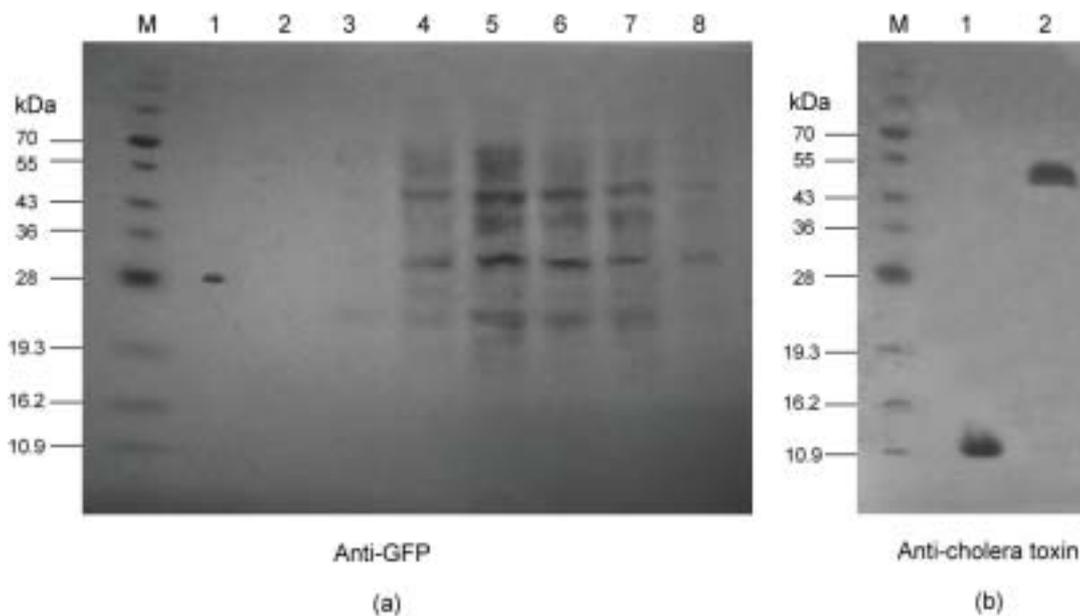


Figure 2.11 Western blotting of fusion antigen protein. The bacterial culture was diluted to  $OD_{660} = 1.0$ , and then 10 ml of cells were pelleted by centrifugation and resuspended in 50  $\mu$ l of  $2 \times$  SDS-PAGE sample buffer. After heating and centrifugation, 15  $\mu$ l of supernatant was loaded in each lane. (a) Time course of expression of antigen protein. Anti-green fluorescent

protein (GFP) antibody. Lane M, protein size marker; lane 1, GFP (MW 28 kDa); lane 2, *B. subtilis* BEST20038 (negative control incubated for 10 h); lanes 3 to 8, *B. subtilis* BEST2131-ctb-cry j 1 epi-gfp (incubation time 6 h, 8 h, 10 h, 12 h, 14 h and 16 h, respectively). (b) Anti-cholera toxin antibody. Lane M, protein size marker; Lane1, cholera toxin B subunit (CTB) (MW 11 kDa); lane 2, *B. subtilis* BEST2131-ctb-cry j 1 epi-gfp (10 h incubation).

The fluorescent characteristics and western blotting analysis of *B. subtilis* containing *ctb-cry j 1 epi-gfp* gene in its genome demonstrated that CTB-Cry j 1 epi-GFP antigen protein was successfully expressed from the gene in the genome of *B. subtilis*.

### **2.3.3 Expression of antigen protein from the antigen gene in the genome and in the plasmid**

The gene integrated in the genome of bacteria has a high stability as compared with the gene in plasmids. However, the amount of antigen protein expression from the gene in the genome of *B. subtilis* is generally lower compared with that from the plasmid because the copy number of the genome of *B. subtilis* is single but that of plasmid in *B. subtilis* is about 9 [57].

In this study, pGETS103 plasmid was used to recovery the *ctb-cry j 1 epi-gfp* antigen gene from the genome of *B. subtilis* by homologous recombination to construct pGETS103-*ctb-cry j 1 epi-gfp* expression vector [12]. Linearized pGETS103 is unable to self-circularize in *B. subtilis* cells, and is therefore unable to replicate. Circular pGETS103 can be established only after recovering the antigen gene from the genome of *B. subtilis* by homologous recombination between the pBR322-derived sequence in the plasmid and at the *B. subtilis* *ctb-cry j 1 epi-gfp* gene insertion locus. The plasmid pGETS103-*ctb-cry j 1 epi-gfp* formed by the recombination was extracted and transformed into *B. subtilis* 168 *trpC2*, which has no locus for homologous recombination.

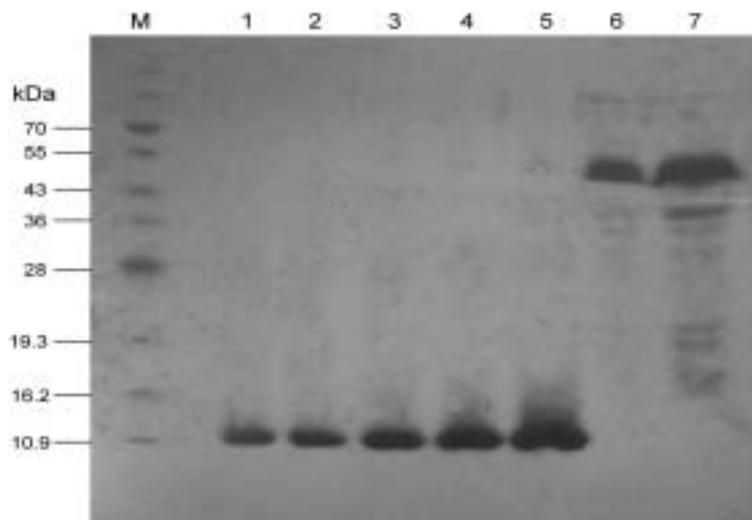


Figure 2.12 Comparison of the yield of fusion antigen protein expressed in *B. subtilis*. Anti-cholera toxin antibody was used. Lane M is protein size marker; lanes 1 to 5 are 20 ng, 40 ng, 80 ng, 160 ng and 240 ng cholera toxin B subunit, respectively (MW 11 kDa); lane 6 is *B. subtilis* BEST2131-ctb-cry j 1 epi-gfp (10 h incubation); lane 7 is *B. subtilis* 168 *trpC2* harboring pGETS103-ctb-cry j 1 epi-gfp plasmid (10 h incubation). Bacteria were pelleted as described for Figure 2.11 and 15  $\mu$ l aliquots were loaded in lanes 6 and 7.

**Figure 2.12** shows the amounts of the fusion antigen expressed from the gene in the genome of *B. subtilis* BEST2131 and from the pGETS103-ctb-cry j 1 epi-gfp in *B. subtilis* 168 *trpC2*. Bandscan version 4.30 (Proenzyme, San Leandro, CA) was used to analyze the amount of expressed antigen protein. The yield of CTB-Cry j1-GFP antigen protein expressed from the genomically-integrated gene was 0.06  $\mu$ g protein/ml of culture (bacterial concentration,  $OD_{660} = 2.0$ ) and the yield from the plasmid was 0.43  $\mu$ g protein/ml of culture ( $OD_{660} = 2.0$ ). The total protein concentration in the culture ( $OD_{660} = 2.0$ ) was 580  $\mu$ g protein/ml. Therefore, genomically-expressed antigen and plasmid-expressed antigen represent approximately 0.01 % and 0.07 % of total protein, respectively.

## 2.4 Conclusions and Discussions

The production of edible vaccines by plants is a significant innovation since plant-derived vaccines offer the advantages of low cost and easy control of

production scale [62, 63]. However, the long growth period and the possibility of gene diffusion to surrounding plants by pollination limit the application of antigen-producing transgenic plants. Therefore, expression of vaccines in edible bacteria is an attractive alternative because of their easy cultivation and short harvesting time.

At present, lactic acid bacteria (LAB) are employed extensively as live carriers of vaccine antigens because the bacteria have no potential risk to humans or animals. When given by the oral route, such live vaccines can induce both mucosal and systemic immune responses to heterologous antigens [49, 50, 64]. The use of genetically modified bacterial strains that contain the antigen gene in their genomes would enhance the stability of gene expression. However, there is little evidence whether genetically modified LAB can be used as live carriers of vaccine antigens. *B. subtilis* has been used in the production of heterologous antigens due to the availability of several well-established expression systems and the ability to secrete recombinant protein into the culture medium. Ivins *et al.* [65] were the first to use *B. subtilis* to express protective antigen for the production of an anti-anthrax acellular vaccine. The use of genetically modified *B. subtilis* strains as live carriers of vaccine antigen has been recently reported. Isticato *et al.* [51] integrated the tetanus toxin fragment C gene of *Clostridium tetani* into the *B. subtilis* genome and the antigen was expressed on the surface of *B. subtilis* spores using a spore coat protein. Later, the B subunit of the heat-labile toxin of *E. coli* was also expressed on the surface of *B. subtilis* spores [66]. Ohashi *et al.* [12] integrated the GFP gene into the *B. subtilis* genome so that GFP protein was stably maintained. Therefore, *B. subtilis* is a good candidate for the stable expression of antigen from genomically-integrated genes, and for use as an edible vaccine due to its potential to incorporate *B. subtilis* (natto) genome to produce hybrid Bacillus.

Japanese cedar pollinosis is one of the major allergic diseases in Japan [18]. Allergen-specific immunotherapy is safer and more effective than conventional immunotherapy for the treatment of IgE-mediated allergic diseases [13-15]. Peptide vaccines using T-cell epitopes would be an effective and safe immunotherapy for

allergic diseases, since recombinant antigen peptides can be designed by selecting T-cell epitopes lacking IgE-binding activity [15]. Recently, Yang *et al.* [5] divided full-length Cry j 1 into three overlapping sub-regions to reduce IgE-binding activity, based on the assumption that most IgE epitopes are conformation-dependent. The three partial genes, Cry j 1 *epi*-1-144, Cry j 1 *epi*-126-157 and Cry j 1 *epi*-231-353, were all fused with the rice storage protein glutelin to construct three fusion antigens. However, the recombinant allergen resulted in a low level of specific IgE-binding activity.

In this study, five major T-cell epitopes from human Cry j 1 because (1) more than 50 % of T-cell lines from pollinosis patients recognized these peptides as T-cell epitopes, and (2) these peptides were presented to T-cells by different HLA class II molecules such as DQ6, DR51, DP5, DR53 and DR15, which are found frequently in the Japanese population [21]. In addition, these T-cell epitopes show no specific IgE-binding activity. The genomically-integrated transgene produced protein within 10 h, and the production scale can be easily controlled. The yield of protein obtained was a little lower than the yields of other recombinant proteins from *B. subtilis* [67]. The yield of plasmid-expressed antigen was approximately seven-fold greater than genomically-expressed antigen which represented only 0.01 % of total bacterial protein. Possible causes of low expression from the integrated transgene are: (1) the single copy compared with the multiple copies of the plasmid and (2) suppression of the expression of the inserted gene. In addition, proteases greatly affected the accumulated amount of the recombinant antigen. To circumvent these problems in later experiments, the following modifications could be tested: (1) the use of a suitable secreted protein for translocation of expressed antigen proteins outside the cell, (2) integration of tandemly repeated antigen genes into the bacterial genome, or integration at multiple loci, (3) use of a suitable fusion partner to increase the amount of expressed antigen protein or (4) the use of protease-deficient strains and the study on the optimal expression conditions to decrease the amount of degradation. The complex regulatory mechanisms of the expression of foreign proteins should be investigated.

# CHAPTER 3

## Expression of Fusion Protein Containing Ovalbumin T-cell Epitopes on *Bacillus subtilis*

### 3.1 Introduction

Heterologous display of proteins on the surface of bacteria is one of useful methods to develop vaccine delivery systems which are able to provide better levels of immunity against pathogens. *B. subtilis*, as one of Gram-positive bacteria, offers several advantages as described in Chapter 2, therefore, I attempted to develop a cell surface display system for the production of live recombinant bacteria based on *B. subtilis*. To date, Istickato *et al.* [51] have already expressed the tetanus toxin fragment C (TTFC) of *Clostridium tetani* on the surface of *B. subtilis* spores using CotB protein. As we know, spore needs the long harvest time compared with vegetative cell and the spore must be purified by complex process. These drawbacks limit the application of spore as vaccine delivery vehicles. Therefore, I attempted to construct a vaccine delivery vehicle via display of foreign antigen on the vegetative cell surface of *B. subtilis* using CotB as an anchoring motif.

In this study, ovalbumin (OVA) T cell epitope [68] was employed as an antigen protein since an ovalbumin T cell epitope integrating with MHC II class molecule can induce OVA-specific T-cell response by oral administration. Cholera toxin B subunit (CTB) was employed as a fusion partner because it is a nontoxic and very useful adjuvant and carrier to enhance the induction of mucosal antibody responses to the linked antigen [29]. To improve the antigenicities of OVA T cell epitope, three repeated immunodominant OVA T cell epitope (OVA<sub>323-339</sub>) were linked to construct OVA epi. Considering lower plasmid copy number in *B. subtilis* than that in *E. coli*, I attempted to construct an expression vector using pHY300PLK plasmid which can shuttle between *E. coli* and *B. subtilis*. To get the high level of protein expression,

middle wall protein (MWP) promoters derived from *Bacillus brevis* 47, powerful multiple promoters for the transcription of mRNA, were employed [69]. In addition, a suitable ribosome binding site (RBS) (AAAGGAGG) [12], and an optimal 9bp distance between RBS and the initiation codon were selected [12].

In this study, three repeated OVA T cell epitope (OVA<sub>323-339</sub>) gene linked to CTB gene and CotB gene linked to the sequence of middle wall protein (MWP) promoters were inserted into pHY300PLK shuttle vector respectively to construct pHY300-mwp-cotB-ctb-ova epi expression vector which was used to transform *B. subtilis* strains. The result of western blotting analysis displayed that CotB-CTB-OVA epi was successfully expressed in *B. subtilis*. The location of expressed protein on the surface of bacteria was confirmed by fluorescent microscopy using anti-cholera toxin antibody and the secondary antibody labeled with a fluorescent dye. Additionally, flow cytometry was used to confirm and quantitatively analyze the cell-surface-displayed CTB-OVA epi using anti-cholera toxin antibody and the 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 Cot B anchoring protein.

## **3.2 Materials and Methods**

### **3.2.1 Bacterial strains, plasmids, growth conditions, transformation**

*Escherichia coli* BL21 (DE3), *E. coli* JM109, pUC18 and pET28a plasmids purchased from Takara Bio (Otsu, Japan) were used for the cloning and the expression of antigen genes, and were grown in Luria-Bertani (LB) medium at 37 °C. Ampicillin (50 µg/ml), kanamycin (25 µg/ml), or tetracycline (10 µg/ml) was used as the selection antibiotic for transformed *E. coli* competent cells. Plasmid of pHY300 was purchased from Takara Bio (Otsu, Japan). *E. coli* JM109 was used for cloning a 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 *B. subtilis* competent cells.

Transformations of *E. coli* JM109 with a recombinant plasmid of pET28a and recombinant plasmids of pHY300 were performed by CaCl<sub>2</sub> method. Transformations of *B. subtilis* 168 *trpC2* with recombinant plasmids of pHY300 were performed by the electroporation method [58].

Ampicillin was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Kanamycin and tetracycline was purchased from Wako (Wako, Osaka, Japan).

### 3.2.2 Construction of fusion antigen genes

#### 3.2.2.1 Construction of *ova epi* antigen gene

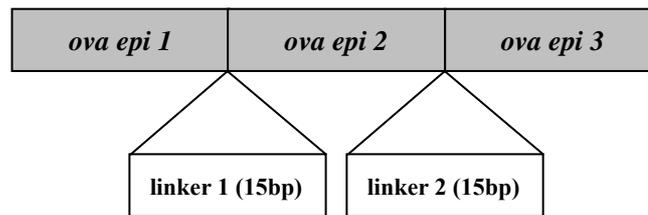


Figure 3.1 Schematic structure of *ova epi* antigen gene

The fusion gene of *ova epi* (*ova epi1-ova epi2-ova epi3*) was constructed by linking three repeated OVA<sub>323-339</sub> sequence using overlap extension PCR method (**Figure 3.1**). The forward primer OVA-1 (5'ATTAGCCAGGCGGTGCATGCGGCGCATGCGGA AATTAATGAAGCGGGCCGCAGCATTTCGCGGCTGG 3', the sequence underlined is the sequence of linker 1) and the reverse primer OVA-2 (3'TCGTAAGCGCCGAC CTAGAGAGTTCGGCAGGTACGTCGCGTACGGCTTTAGTTACTCCGTCCCGC A5', the sequence underlined is complementary to the 3' end sequence of primer OVA-1(the sequence of linker 1) 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 (5' ATTAGCCAGGCGGTG3') consisting

of the first 15bp of the 5' end sequence of primer OVA-1 and the reverse primer OVA-4 (3'TTACTCCGTCCCGCACCGTAAACCAGAGCG5', the sequence underlined is the first 15bp of the 5' end sequence of reverse primer OVA-2, the sequence indicated in bold is the sequence of linker 2) were added to amplify the PCR product *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 (3'CCGTAAACCAGAGCGTAAAGGGTCCGTCAAGTACGGCGCGTACGTCTTTAGTTGCTCCGGCCAGCG5', the sequence underlined is the first 15bp of the 5' end sequence of reverse primer OVA-4 (the sequence of linker 2 )) 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. The forward primer OVA-3 (5'ATTAGCCAGGCGGTG3') complementary to the 5' end sequence of *ova epi 1-ova epi 2* fusion gene and the reverse primer OVA-6 (3'TTGCTCCGGCCAGCGTT**CGA**AAGCG5', the sequence underlined is the first 15bp of the 5' end sequence of primer OVA-5, the sequence indicated in bold is the *Hind* III site) were added to amplify the final PCR product *ova epi (ova epi 1-ova epi 2-ova epi 3)* fusion antigen gene. All the PCR products were extracted and purified by Gel Extraction Kit (Qiagen, Tokyo, Japan). All the primers were purchased from Invitrogen Japan (Tokyo, Japan).

### 3.2.2.2 Construction of *ctb-ova epi* fusion antigen gene

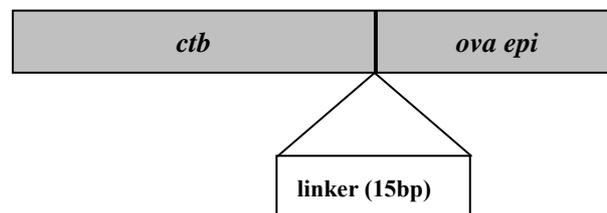


Figure 3.2 Schematic structure of *ctb-ova epi* antigen gene

The C-terminus ending of CTB was fused to OVA T-cell epitopes via a KRWLV linker (**Figure 3.2**). 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 of the first PCR. The sequence of linker was linked to *ctb* by PCR method using the forward primer (5'ACACCTCAAATATTACTG3') complementary to the 5' end sequence of *ctb* and the reverse primer (3'*TAATCATA*CCGTTTATTGCGACCGACCAC5', the sequence indicated in italic is complementary to the 3' end of *ctb* and the sequence underlined is the linker sequence). KOD-plus DNA polymerase was used to amplify *ctb-linker* fusion gene. Then, using *ctb-linker* fusion gene as the template, PCR was performed to link the 5' end sequence of *ova epi* to the 3' end of *ctb-linker* gene. The forward primer (5'-ACACCTCAAATATTACTG-3') complementary to the 5' end sequence of *ctb* gene and the reverse primer (3'TTTGCGACCGACCACTAATCGGTCCGCCAC5', the sequence underlined is complementary to the 3' end sequence of *ctb-linker* and the sequence indicated in italic is the first 15bp of the 5' end sequence of *ova epi*) were used. Finally, *ctb-linker-ova epi* was constructed using overlap extension PCR method again. The two PCR products, *ctb-linker-ova epi* (containing 15bp 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 (5'GCGGGATCCATGACACCTCAAATATTACTGAT3', the sequence indicated in bold is the *Bam* HI site, the sequence indicated in italic is the start codon, and the sequence underlined is complementary to the 5' end sequence of *ctb* gene) and the reverse primer (5'GCGAAGCTTGCGACCGGCCTCGTT 3', the sequence indicated in bold is the *Hind* III site, and the sequence underlined is complementary to the 3' end sequence of *ova epi* fusion antigen gene) were added to amplify the final PCR product *ctb-ova epi* fusion antigen gene.

### 3.2.2.3 Construction of *mwp*-RBS fusion gene

To get the 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* [12]. This corresponded to the experimental data that the optimal RBS was AAAGGAGG and

the optimal distance between RBS and start codon was 7-9 nucleotides [12]. The forward primer MWP-Fw (5'AACTTGGCTGTTGTAAACTTTGAAAATGC3') and the reverse primer MWP-RBS-Rv (5'**CCTCCTTTCGCAGGAAAGCCTCGCTTTTT** G3', the sequence indicated in bold is RBS sequence, and the sequence indicated in italic is complementary to the 3' end sequence of MWP promoters) were used to amplify the PCR product *mwp*-RBS from the pUC18 plasmid containing MWP promoters. This plasmid was constructed by integrating the sequence of MWP promoters amplified by PCR from the genome of *Bacillus brevis*.

### 3.2.2.4 Construction of *mwp*-RBS-*cotB* fusion gene

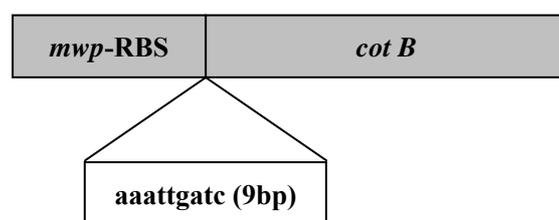


Figure 3.3 Schematic structure of *mwp*-RBS-*cotB* antigen gene for integration into pHY300

Using the *mwp*-RBS fusion gene constructed as the template, the 5' end sequence of CotB gene (*cotB*) was linked to the 3' end of *mwp*-RBS. The forward primer MWP-Fw (5'AACTTGGCTGTTGTAAACTTTGAAAATGC3') and the reverse primer MWP-RBS-*cotB*-Rv (5'CATTCTCCTCTTGCTCAT**gatcaattt***CCTCCTTTCGCAGGAAAG*3', the sequence underlined is complementary to the 5' end sequence of *cotB* gene, the sequence indicated in lowercase is a 9-nucleotide-spacing between RBS and start codon, the sequence indicated in bold is RBS sequence, and the sequence indicated in italic is complementary to the 3' end sequence of MWP promoters) were used to amplify the PCR product *mwp*-RBS-*cotB* (containing 15bp of the 5' end sequence of *cotB*) fusion gene.

To construct the *mwp*-RBS-*cotB* fusion gene, *mwp*-RBS-*cotB* (containing 15bp of the 5' end sequence of *cotB*) was linked to *cotB* by overlap extension PCR. The CotB

gene sequence was amplified from the genome of *B. subtilis* 168 by the forward primer CotB-Fw (5'**ATGAGCAAGAGGAGAATGAAATATCAT**3', the sequence indicated in bold is the start codon) and the reverse primer CotB-Rv (5'GGATGAT TGATCATCTGAAGATTTTAG3'). Two PCR products, *mwp*-RBS-*cotB* (containing 15bp of 5' end sequence of Cot B gene) and *cot B* 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 (5'AACTTGGCTGTTGTAAACTTTGAAAATG C3') and the reverse primer CotB-Rv (5'GGATGATTGATCATCTGAAGATTTTA G3') were added to amplify the final PCR product *mwp*-RBS-*cotB* fusion gene (**Figure 3.3**). Taq DNA polymerase (Takara Bio, Shiga, Japan) was used for this PCR to add one A nucleotide at 3'-termini of the PCR product for the following ligation with the T vector.

### 3.2.3 Construction of recombinant plasmid for *E. coli*

The DNA fragment of *ctb-ova epi* amplified by PCR was digested with *Bam* HI and *Hind* III restriction enzymes and cloned into corresponding sites of pUC18 plasmid to produce recombinant cloning plasmids of pUC18-*ctb-ova epi*. *E. coli* JM109 competent cells were transformed with the pUC18-*ctb-ova epi* recombinant plasmids, and positive colonies were selected from LB plates containing 100µg/ml ampicillin. Inserted DNA fragment of *ctb-ova epi* was digested out of extracted plasmids with *Bam*HI and *Hind* III restriction enzymes and then inserted into pET28a expression plasmid, which provides T7 promoter and T7 terminator, to construct recombinant expression plasmids of pET28a-*ctb-ova epi*. **Figure 3.4** shows the structure of recombinant pET28a recombinant plasmid. All the enzymatic digestion products were purified using Gel Extraction Kit (Qiagen, Tokyo, Japan).



pAM-alpha of *Streptococcus faecalis*. It contains the tetracycline resistance (Tc) gene of pAM $\alpha$ 1 and its replication origins, *ori*  $\alpha$ , and the ampicillin resistance (Amp) gene of pACYC177 and its replication origins, *ori* 177 so that it can shuttle between *E.coli* and *B. subtilis*. **Figure 3.5** shows the structure of pHY300 vector.

#### 3.2.4.1 Construction of pHY300-mwp-ctb-ova epi recombinant plasmid

To construct an expression vector which can shuttle between *E. coli* and *B. subtilis*, the antigen gene, *ctb-ova epi*, was digested out of pUC18-ctb-ova epi plasmid with *Bam*HI and *Hind* III restriction enzymes and then cloned into pHY300 vector digested in corresponding sites to form pHY300-ctb-ova epi recombinant plasmid. The sequence of *mwp*-RBS amplified by PCR using Taq polymerase was cloned into pHY300-ctb-ova epi recombinant plasmid by T-A cloning method as described below at the *Sma*I site close to *Bam*HI site (5'CCCGGGGATCC3', the sequence underlined is *Sma*I site and the sequence in bold is *Bam*HI site) (**Figure 3.6**). T vector of pHY300-ctb-ova epi was prepared according to the method described by Ohashi *et al.* [12]. Two  $\mu$ g of pHY300-ctb-ova epi was digested completely with 30U of *Sma*I at 30 °C for 6h and treated with phenol/chloroform extraction. After precipitation with ethanol, DNA was dissolved in 10 $\mu$ l of TE buffer. An aliquot of 5 $\mu$ l of the digested plasmid solution was treated with *Ex Taq* DNA polymerase (Takara, Otsu, Japan) at a ratio of 2.5U/ $\mu$ g plasmid in 50 $\mu$ l buffer solution (1  $\times$  *Ex Taq* buffer supplemented with 2mM dTTP) at 72 °C for 2h. After phenol/chloroform extraction twice and isopropanol precipitation, the T-vector was dissolved in 10 $\mu$ l of TE buffer and stored at -80 °C until use.

The sequence of *mwp*-RBS amplified by PCR using Taq polymerase was introduced into the T-vectors at 16 °C overnight using DNA Ligation Kit (Takara, Otsu, Japan) as described in the manual. **Figure 3.6** shows the structure of pHY300-mwp-ctb-ova epi recombinant expression plasmid. **Figure 3.7** shows the structure of the fusion gene inserted into recombinant plasmid.

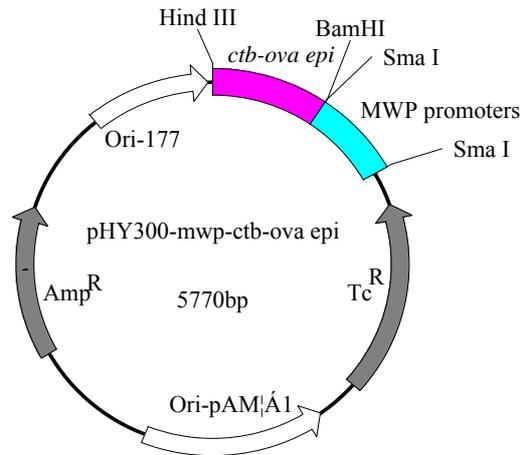


Figure 3.6 Schematic structure of pHY300-mwp-ctb-ova epi expression vector

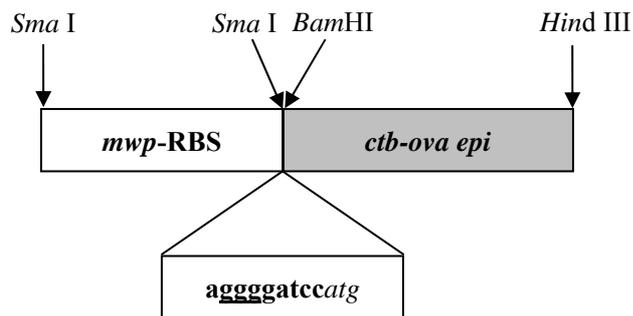


Figure 3.7 Schematic structure of *ctb-ova epi* antigen gene for integration into pHY300

The pHY300-mwp-ctb-ova epi recombinant plasmid was amplified in *E. coli* JM109 described below. Ten  $\mu$ l of ligation solution was incubated with 100 $\mu$ l of *E. coli* JM109 competent cells for 30min on ice, followed by incubation at 42 °C for 45sec. After 2min on ice, 1ml SOC medium was added. *E. coli* was grown at 37 °C under shaking at 200rpm for 1h. One hundred and fifty  $\mu$ l of the culture was spread on LB plate supplemented with an appropriate antibiotic, followed by incubation at 37 °C overnight. Since there were two possible orientations of the sequence of

*mwp*-RBS inserted in the T-extended *Sma*I ends of pHY300-*ctb-ova* epi, forward primer pHY300-Fw (5'GCGCCTATGGAAGTTGATCAGTCAACTT3') and reverse primer MWP-RBS-RV (5'**CCTCCTTT***CGCAGGAAAGCCTCGCTTTTTG*3', the sequence indicated in bold is RBS sequence, and the sequence indicated in italic is complementary to the 3' end sequence of MWP promoters) were used to confirm the desired orientation of inserted gene in the recombinant plasmid by colony PCR.

Recombinant plasmid of pHY300-*mwp-ctb-ova* epi from *E. coli* JM109 was extracted using Spin Miniprep Kit (Qiagen, Tokyo, Japan) as described in the manual. DNA sequences inserted in the plasmid were checked using the dye-terminator method of Bio Matrix Research (Nagareyama, Japan).

### 3.2.4.2 Construction of pHY300-*mwp-cotB-ctb-ova* epi recombinant plasmid for cell surface display

To construct a surface display expression vector, the sequence of *mwp-RBS-cotB* amplified by PCR using Taq polymerase was cloned into pHY300-*ctb-ova* epi recombinant plasmid by T-A cloning method as described in 3.2.4.1. **Figure 3.8** shows the structure of pHY300-*mwp-cotB-ctb-ova* epi recombinant plasmid. **Figure 3.9** shows the structure of the gene inserted into recombinant plasmid.

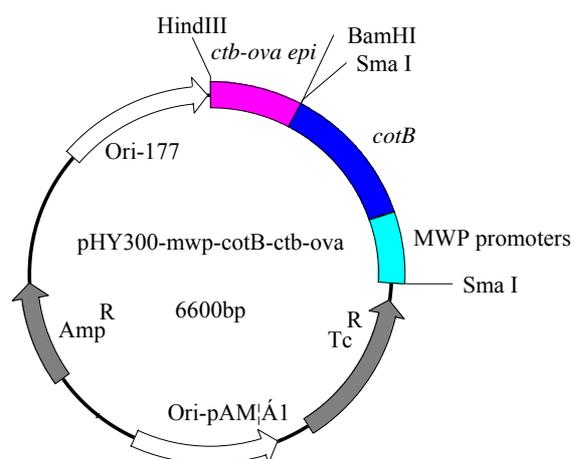


Figure 3.8 Construction of pHY300-*mwp-cotB-ctb-ova* epi surface display expression vector

The pPHY300-mwp-cotB-ctb-ova epi recombinant plasmid was also amplified in *E. coli* JM109 described in 3.2.4.1. To confirm the desired orientation of the sequence of *mwp*-RBS-*cotB* inserted in the T-extended *Sma*I ends of pPHY300-ctb-ova epi, forward primer pPHY300-Fw (5'GCGCCTATGGAAGTTGATCAGTCAACTT3') and reverse

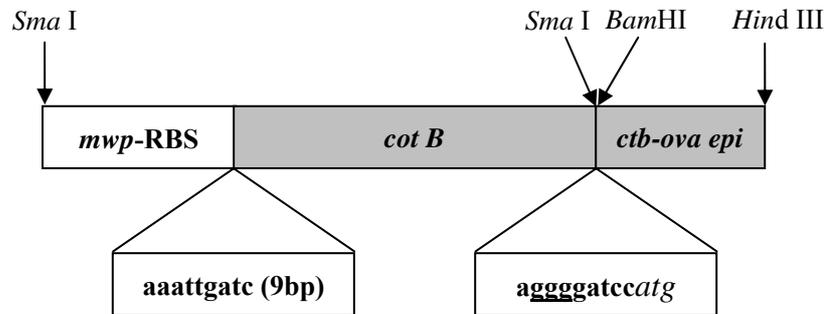


Figure 3.9 Schematic structure of *cotB-ctb-ova epi* antigen gene for integration into pPHY300

primer MWP-RBS-*cotB*-Rv (5'CATTCTCCTCTTGCTCAT**gatcaattt**CCTCCTTTC GCAGGAAAG3'), the sequence underlined is complementary to the 5' end sequence of *cotB* gene, the sequence indicated in lowercase is a 9-nucleotide-spacing between RBS and start codon, the sequence indicated in bold is RBS sequence, and the sequence indicated in italic is complementary to the 3' end sequence of MWP promoters) were used to confirm the desired orientation of the inserted gene in the recombinant plasmid by colony PCR.

Recombinant plasmid of pPHY300-mwp-ctb-*cotB*-ova epi from *E. coli* JM109 was extracted using Spin Miniprep Kit (Qiagen, Tokyo, Japan) as described in the manual. DNA sequences inserted into the plasmid were checked using the dye-terminator method of Bio Matrix Research.

### 3.2.5 Expression of antigen protein in *E. coli*

*E. coli* BL21 (DE3) competent cells were transformed with the pET28a-ctb-ova epi expression plasmid. The positive colonies were selected with kanamycin (25 µg/ml). A single positive colony was grown in 2ml of LB medium containing kanamycin (25 µg/ml) under shaking at 37 °C for 6 h. Protein expression was then induced with 1mM IPTG and the bacteria were further cultured at 37 °C for 12 h. Cells were pelleted by centrifugation at 6,000×g for 5 min at 4 °C and then resuspended in 0.5ml of 20mM Tris-HCl (pH 8.0). The suspension was mixed with 2×SDS-PAGE sample buffer. After heating for 5min at 90 °C, 15µl of supernatant was loaded in each lane of SDS-PAGE gel, followed by western blotting to detect the expression of the antigen protein.

### 3.2.6 Expression of antigen protein in *B. subtilis*

*B. subtilis* 168 *trpC2* competent cells were transformed with recombinant plasmids of pHY300 by electroporation method as described in 2.2.5.5. *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 600M (Gentronics, San Diego, CA) at a voltage of 2.5 kV and a resistance of 186 ohms. The positive colonies were selected with tetracycline (10 µg/ml).

To confirm the expression of antigen protein in *B. subtilis* 168 *trpC2*, a single positive colony of *B. subtilis* 168 *trpC2* transformed by pHY300-mwp-ctb-ova epi and pHY300-mwp-cotB-ctb-ova epi were precultured in 2 ml of LB medium containing chloramphenicol (5 µg/ml) at 37 °C for 12 h under shaking (200 rpm). Then, 50 µl each of preculture was added in six tubes containing 5 ml LB medium and 5 µg/ml of chloramphenicol and incubated at 37 °C for 8 to 18 h under shaking (250 rpm). The concentration of cells was monitored at 660 nm on a UV spectrophotometer mini 1240 (Shimadzu, Japan), and equal number of cells were pelleted by centrifugation at 15,000 rpm for 5 min at 4 °C and resuspended in 50 µl of 2×SDS-PAGE sample buffer. The suspensions were heated for 5 min at 90 °C. After centrifugation at 15,000 rpm for 5 min at 4 °C, 15µl of supernatant was used for

SDS-PAGE, followed by western blotting to detect the expression of the antigen peptide.

### **3.2.7 Western blotting**

Proteins were separated on SDS-PAGE gels and then transferred onto PVDF transfer membrane (Hybond-P) (Amersham Bioscience, Tokyo, Japan) using the semi-dry method [61]. Rabbit anti-cholera toxin antibody (C3062) (Sigma-Aldrich Japan, Tokyo, Japan) was used as the primary antibody for both CTB-OVA epi and CotB-CTB-OVA epi recombinant proteins. The secondary antibody was donkey anti-rabbit antibody conjugated with horseradish peroxidase (NA 934V) (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.

### **3.2.8 Immunofluorescence microscopy**

Immunostaining was performed as follows. *B. subtilis* cells were cultivated in LB medium at 37 °C for 8h, collected by centrifugation at 3,500×g for 5min at 4 °C, and washed with PBS (pH 7.2).

The slide glass was incubated in ethanol for 1 h and rinsed with distilled and deionized water (DDW), followed by incubation in 100 µg/ml poly-L-lysine for 1 h and rinsed with DDW. The slide glass was then sterilized under UV light for 1 h. The pelleted cells were resuspended in PBS and then fixed on the slide glass by intermittent heating at 42 °C. The cells and the primary antibody were incubated in PBS containing 1% bovine serum albumin (BSA) for 1h at room temperature. Rabbit anti-cholera toxin antibody (C 3062) (Sigma-Aldrich Japan, Tokyo, Japan) diluted 2000-fold with PBS was used as the primary antibody. After washing with PBS, the cells were incubated for 1 h at room temperature with the 200-fold diluted second antibody, goat anti-rabbit IgG conjugated with FITC (F 0382) (Sigma-Aldrich Japan, Tokyo, Japan). After washing with PBS, the slide glass was sealed with nail polish, then the cells were observed on a microscope. Immunofluorescence microscopy

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

### 3.2.9 Flow cytometry

One hundred  $\mu$ l of *B. subtilis* suspension in PBS ( $OD_{600}= 0.02$ ) was stained with the same volume of the 2,000-fold diluted rabbit anti-cholera toxin antibodies (Sigma C3062) for 20 min on ice. Cells were then washed with PBS and stained with 100  $\mu$ l of 100-fold diluted FITC-labeled goat anti-rabbit IgG (Sigma F0382). Flow cytometry was optimized for the analysis of bacteria by raising the FSC (forward scatter) voltage to E03 (FACScan, Becton-Dickinson Japan, Tokyo). Fifty thousands of particles were acquired in a logarithmic fluorescence mode.

## 3.3 Results

### 3.3.1 Construction of fusion antigen genes

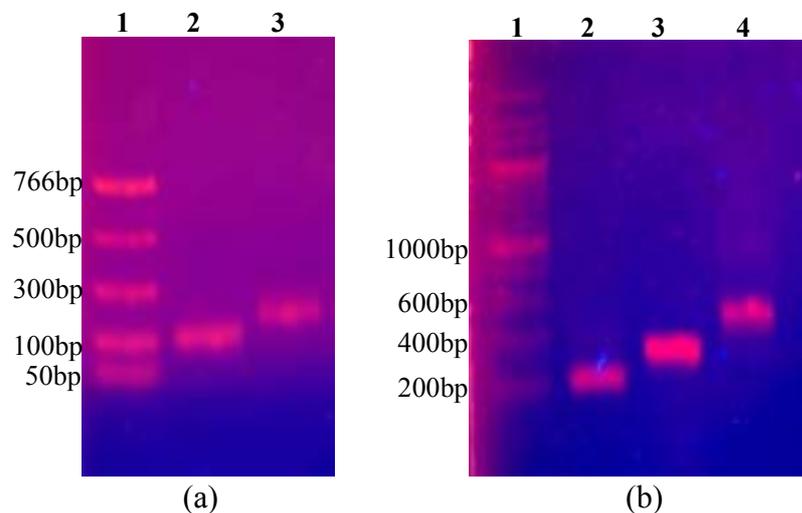


Figure 3.10 Electrophoretic analysis of PCR products. (a) Lane 1, 50bp DNA ladder; Lane 2, *ova ep 1-ova epi 2* fusion gene (117bp); Lane 3, *ova ep 1-ova epi 2-ova epi3* (*ova epi*) fusion antigen gene (183bp). (b) Lane 1, 200bp DNA ladder; Lane 2, *ova epi* fusion gene (183bp); Lane 3, *ctb* gene (309bp). Lane 3, *ctb-ova epi* fusion antigen gene (510bp).

Fusion antigen gene, *ctb-ova epi*, was constructed by overlap extension PCR as described in Materials and Methods. The structure of *ctb-ova epi* antigen gene is shown in **Figure 3.1**. Electrophoretic analysis of PCR products was shown in **Figure 3.10**. Results of sequencing analysis showed that DNA and deduced amino acid sequences were identical to the designed ones.

### 3.3.2 Construction of recombinant plasmid for *E. coli*

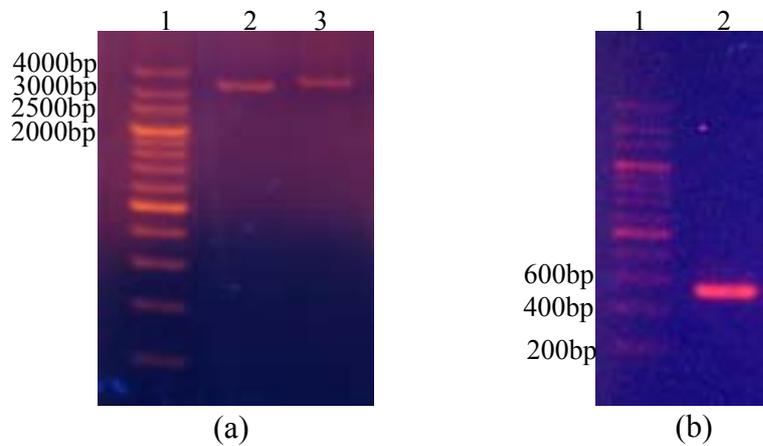


Figure 3.11 Electrophoretic analysis of recombinant plasmids and a PCR product. (a) Lane 1, 200bp DNA ladder; Lane 2, pET28a (5369bp); Lane 3, pET28a-*ctb-ova epi* (5850bp); (b) Lane 1, 200bp DNA ladder; Lane 2, *ctb-ova epi* fusion antigen gene amplified from pET28a-*ctb-ova epi* (510bp).

Fusion antigen gene of *ctb-ova epi* was inserted into pET28a plasmid using double enzyme method as described in 3.2.3 to construct pET28a-*ctb-ova epi* recombinant plasmid. **Figure 3.11** shows the result of electrophoretic analysis of recombinant plasmid and its PCR product. The sequence of *ctb-ova epi* fusion gene inserted in pET28a plasmid was confirmed by Bio Matrix Research. Results of sequencing analysis showed that DNA and deduced amino acid sequences were identical to the designed ones (**Figure 3.14**, **Figure 3.15**).

### 3.3.3 Construction of recombinant plasmid for *B. subtilis*

#### 3.3.3.1 Construction of pHY300-mwp-ctb-ova epi recombinant plasmid

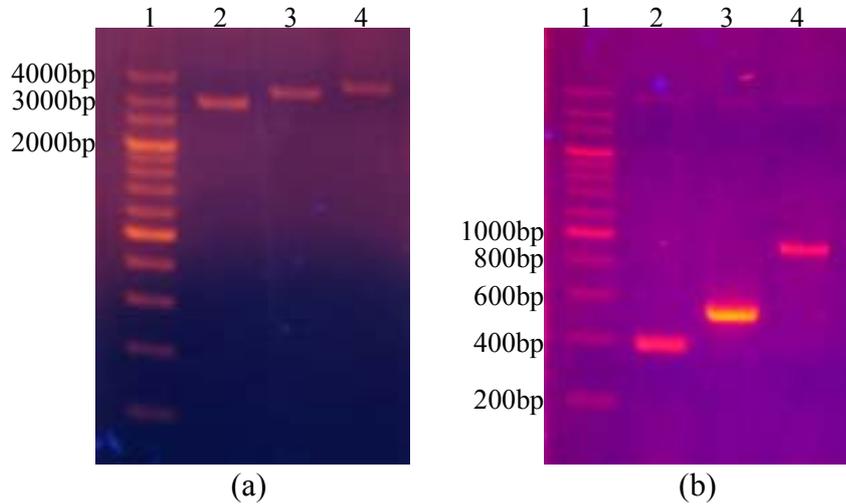


Figure 3.12 Electrophoretic analysis of recombinant plasmids and PCR products (a) Lane 1, 200bp DNA ladder; Lane 2, pHY300 (4870bp); Lane 3, pHY300-ctb-ova epi (5350bp); Lane 4, pHY300-mwp-ctb-ova epi (5748bp); (b) Lane 1, 200bp DNA ladder; Lane 2, mwp (381bp); Lane 3, *ctb-ova epi* fusion antigen gene (510bp); Lane 4, mwp-*ctb-ova epi* fusion antigen gene (906bp). All the PCR products were amplified from pHY300-mwp-ctb-ova epi recombinant plasmid.

Fusion antigen gene of *ctb-ova epi* for insertion into pHY300 plasmid for the expression of antigen was constructed by overlap extension PCR. The structure of *mwp-ctb-ova epi* antigen gene is shown in **Figure 3.7**. **Figure 3.12** shows the result of electrophoretic analysis of recombinant plasmids and PCR products amplified from the plasmids. The sequence of mwp-ctb-ova epi fusion gene inserted in pHY300 plasmid was confirmed by Bio Matrix Research. Results of sequencing analysis showed that DNA and deduced amino acid sequences were identical to the designed ones (**Figure 3.14**, **Figure 3.15**).

### 3.3.3.2 Construction of pHY300-mwp-cotB-ctb-ova epi recombinant plasmid

Fusion antigen gene of *ctb-ova epi* was inserted into pHY300 plasmid by double enzyme method to construct pHY300-ctb-ova epi recombinant plasmid. Then, the DNA sequence of MWP promoters and RBS was integrated into pHY300-ctb-ova epi

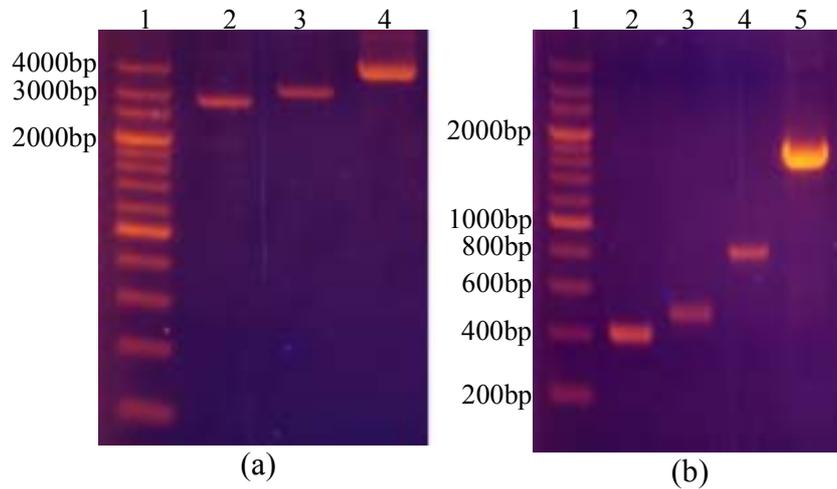


Figure 3.13 Electrophoretic analysis of recombinant plasmids and PCR products. (a) Lane1, 200bp DNA ladder; Lane 2, pHY300 (4870bp); Lane 3, pHY300-ctb-ova epi (5350bp); Lane 4, pHY300-mwp-cotB-ctb-ova epi (6600bp); (b) Lane 1, 200bp DNA ladder; Lane 2, mwp (381bp); Lane 3, *cotB* gene (825bp); Lane 4, mwp-cotB-ctb-ova epi fusion antigen gene (1735bp). All the PCR products were amplified from pHY300-mwp-cotB-ctb-ova epi recombinant plasmid.

recombinant plasmid by T-A cloning method to construct pHY300-mwp-ctb-ova epi recombinant expression plasmid. The structure of *mwp-ctb-ova epi* is shown in **Figure 3.9**. **Figure 3.13** shows the result of electrophoretic analysis of recombinant plasmids and PCR products. The sequence of mwp-ctb-ova epi inserted in pHY300 plasmid was confirmed by Bio Matrix Research. Results of sequencing analysis showed that DNA and deduced amino acid sequences were identical to the designed ones (**Figure 3.14**, **Figure 3.15**).

(a) DNA sequence of MWP promoters gene

AACTTGGCTGTTGTAAACTTTGAAAATGCATTAGGAAATTAACCTAATTCAA  
GCAAGATTATGAGGTTTTGAACCAAATTGGAAAAAGGTTTCAGTCGTGACA  
GCCCCCATATGTCCCCTATAATACGGATTGTGGCGGATGTCACTTCGTACAT  
AATGGACAGGTGAATAACGAACCACGAAAAAACTTTAAATTTTTTTTCGAA  
GGCGCCGCAACTTTTGATTTCGCTCAGGCGTTTAATAGGATGTCACACGAAA  
AACGGGGAATTGTGTAAAAAAGATTCACGAATTCTAGCAGTTGTGTTACAC  
TAGTGATTGTTGCATTTTACACAATACTGAATATACTAGAGATTTTTAACACA  
AAAAGCGAGGCTTTCCTGCG (381bp)

(b) DNA sequence of CotB-CTB-OVA epi antigen gene

ATGAGCAAGAGGAGAATGAAATATCATTCAAATAATGAAATATCGTATTATA  
ACTTTTTGCACTCAATGAAAGATAAAATTGTTACTGTATATCGTGGAGGTCC  
GGAATCTAAAAAAGGAAAATTAACAGCTGTAAAATCAGATTATATAGCTTTA  
CAAGCTGAAAAAAAATAATTTATTATCAGTTGGAGCATGTGAAAAGTATTA  
CTGAGGATACCAATAATAGCACCAACAATTGAGACTGAGGAAATGCTCG  
ATGCTGATGATTTTCATAGCTTAATCGGACATTTAATAAACCAATCAGTTCAA  
TTTAACCAAGGGGGTCCGGAATCTAAAAAAGGAAGATTGGTCTGGCTGGG  
AGATGATTACGCTGCGTTAAACACAAATGAGGATGGGGTAGTGTATTTAAT  
ATCCATCACATCAAAGTATAAGTAAACACGAGCCTGATTTGAAAATAGAA  
GAGCAGACGCCAGTTGGAGTTTTGGAAGCTGATGATTTAAGCGAGGTTTTT  
AAGAGTCTGACTCATAAATGGGTTTCAATTAATCGTGGAGGTCCGGAAGCC  
ATTGAGGGTATCCTTGTAGATAATGCCGACGGCCATTATACTATAGTGAAAA  
ATCAAGAGGTGCTTCGCATCTATCCTTTTCACATAAAAAGCATCAGCTTAGG  
TCCAAAAGGGTCGTACAAAAAAGAGGATCAAAAAAATGAACAAAACCAG  
GAAGACAATAATGATAAGGACAGCAATTCGTTCAATTTCTTCAAATCATATA  
GCTCATCAAATCATCTAAACGATCACTAAAATCTTCAGATGATCAATCATC  
CAGGGGATCCATGACACCTCAAATATTACTGATTTGTGTGCAGAATACCAC  
AACACACAAATACATACGCTAAATGATAAGATATTTTCGTATACAGAATCTCT  
AGCTGGAAAAAGAGAGATGGCTATCATTACTTTTAAGAATGGTGCAACTTT

TCAAGTAGAAGTACCAGGTAGTCAACATATAGATTCACAAAAAAGCGAT  
TGAAAGGATGAAGGATACCCTGAGGATTGCATATCTTACTGAAGCTAAAGT  
CGAAAAGTTATGTGTATGGAATAATAAACGCCTCATGCGATTGCCGCAATT  
AGTATGGCAAATAAACGCTGGCTGGTGATTAGCCAGGCGGTGCATGCGGCGC  
*ATGCGGAAATTAATGAAGCGGGCCGCAGCATTTCGCGGCTGGATCTCTCAAGCC*  
*GTCCATGCAGCGCATGCCGAAATCAATGAGGCAGGGCGTGGCATTGGTCTCG*  
*CATTCCCAGGCAGTTCATGCCGCGCATGCAGAAATCAACGAGGCCGGTTCGCT*  
*AA* (1347bp)

Figure 3.14 DNA sequences of MWP promoters and CotB-CTB-OVA epi fusion antigen. (a) DNA sequence of MWP promoters gene (b) DNA sequence of CotB-CTB-OVA epi antigen gene. The sequence without emphasis indicates CotB gene (825bp), the sequence underlined indicates CTB gene (309bp), the sequence in italic indicates OVA epi (183bp), the sequences in red are start and stop codons, and the sequences in blue are the linkers (15bp × 3).

Amino acid sequence of CotB-CTB-OVA epi fusion antigen

MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSDYIAL  
 QAEKKIIYYQLEHVKSITEDTNNSTTTIETEEMLDADDFHSLIGHLINQSVQFN  
 QGGPESKKGRLVWLGDDYAALNTNEDGVVYFNIHHIKSISKHEPDLKIEEQTP  
 VGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNADGHYTIVKNQEVLR  
 IYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSNSFISSKSYSSSKSSKRSL  
KSSDDQSSRGS-MTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAITF  
KNGATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPH  
*AIAAISMAN**KRWLVISQAVHAAHAEINEAGRSIRGWISQAVHAAHAEINEAGRSWI*  
*GRISQAVHAAHAEINEAGR* (449bp)

Figure 3.15 Amino acid sequence of CotB-CTB-OVA epi fusion antigen. The sequence without emphasis indicates CotB gene (275a.a), the sequence underlined indicates CTB gene (103a.a), the sequence in italic indicates OVA epi (61bp), and the sequences in blue are the linkers (5a.a × 3).

### 3.3.4 Expression of antigen protein in *E. coli*

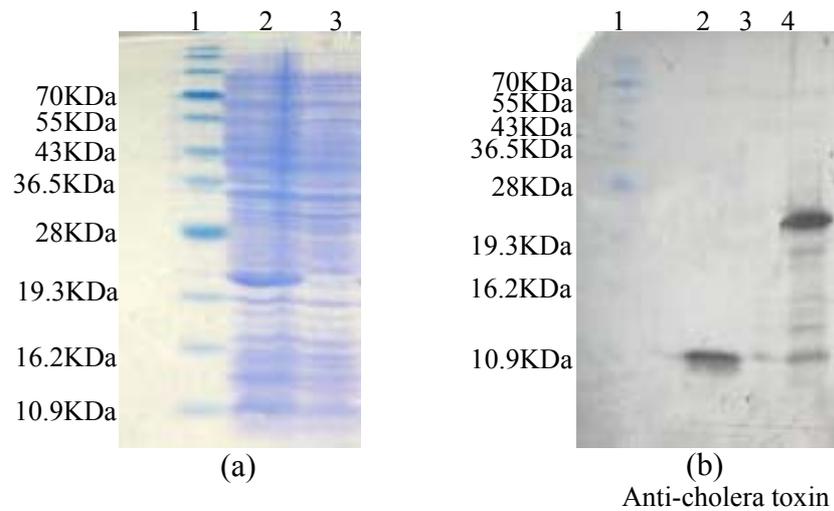


Figure 3.16 SDS-PAGE and western blotting analysis of CTB-OVA antigen expressed in *E. coli*. (a) Lane 1, prestained size marker of protein; Lane 2, *E. coli* BL21(DE3) harboring pET18a-ctb-ova epi recombinant plasmid induced with IPTG; Lane 3, *E. coli* BL21(DE3) harboring pET18a-ctb-ova epi recombinant plasmid (no IPTG, negative control). Proteins were stained with coomassie brilliant blue. (b) Lane 1, prestained size marker of protein; Lane 2, Commercial CTB; Lane 3, *E. coli* BL21(DE3) harboring pET18a-ctb-ova epi recombinant plasmid (no IPTG, negative control); Lane 4, *E. coli* BL21(DE3) harboring pET18a-ctb-ova epi recombinant plasmid induced with IPTG. Anti-cholera toxin antibody was used.

Western blotting analysis was performed to analyze the expressed CTB-OVA antigen protein in *E. coli*. CTB-OVA epi antigen (theoretical molecular weight of 23.6 kDa including the peptide sequence coded by pET28a plasmid) was recognized by anti-cholera toxin antibody. **Figure 3.16** shows the result of western blotting analysis. The result indicated that CTB-OVA epi antigen protein was successfully expressed in *E. coli*.

### 3.3.5 Expression of CTB-OVA epi antigen protein in *B. subtilis*

Western blotting was performed to confirm the antigen protein expressed in *B. subtilis* by pHY300-mwp-ctb-ova epi recombinant expression plasmid. **Figure 3.17** shows that a maximal amount of expressed protein corresponding to the theoretical

molecular weight of CTB-OVA epi (18.2 kDa) was observed at 10 h (**lane 3, Figure 3.17**). From **Figure 3.17**, we also found the expressed antigen proteins were degraded with the increase of incubation time. This is probably due to proteases produced in *B. subtilis* because proteins with smaller sizes (about 12kDa) assumed as proteolytic products appear at 10 h and later (**lane 3 to 8, Figure 3.17**). The result of western blotting analysis indicated that the CTB-OVA epi antigen protein was successfully expressed in *B. subtilis* and the pHY300-mwp-ctb-ova epi expression vector is functional in *B. subtilis*.

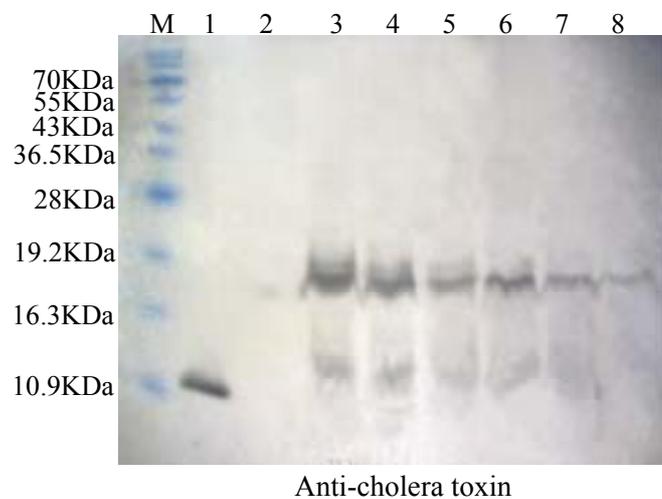


Figure 3.17 Time course of the expression of CTB-OVA antigen protein analyzed by western blotting. Anti-cholera toxin antibody was used. Lane M, prestained size marker of protein; Lane1, commercial CTB (molecular weight, 11 KDa); Lane2, *B. subtilis* 168 harboring pHY300-ctb-ova plasmid (no promoter, negative control); Lanes 3 to 8, *B. subtilis* 168 harboring pHY300-mwp-ctb-ova epi plasmid (incubation time was 10 h, 12 h, 14 h, 16 h, 18 h, and 20 h, respectively).

### 3.3.6 Expression of CTB-OVA epi antigen protein on the surface of *B. subtilis*

#### 3.3.6.1 Expression of CotB-CTB-OVA epi antigen protein in *B. subtilis*

After confirmation of the functionality of pHY300-mwp-ctb-ova epi expression plasmid in *B. subtilis*, the N-terminus ending of CTB-OVA epi antigen peptide was

fused to CotB peptide employed as a fusion partner to display the antigen gene on the surface of *B. subtilis*. Western blotting was performed to confirm the antigen protein

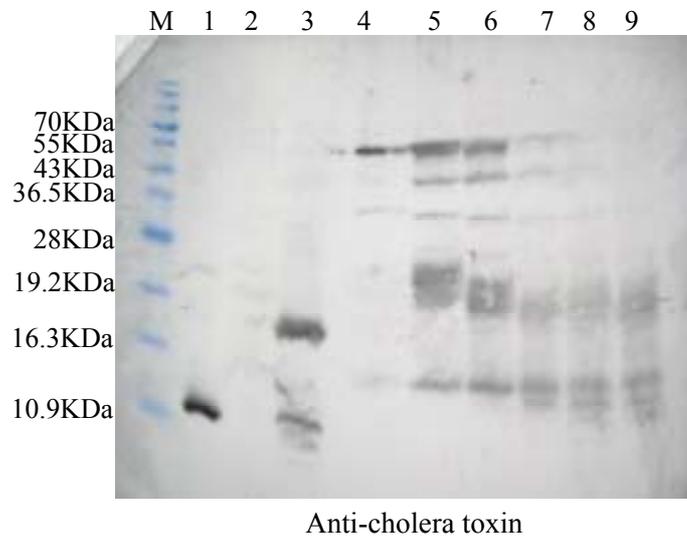


Figure 3.18 Time course of the expression of CotB-CTB-OVA antigen protein analyzed by western blotting. Anti-cholera toxin antibody was used. Lane M, prestained size marker of protein; Lane1, CTB (Molecular weight is 11 KDa); Lane2, *B. subtilis* 168 harboring pHY300-ctb-ova plasmid (no promoter, negative control); Lanes 3, *B. subtilis* 168 harboring pHY300-mwp-ctb-ova epi plasmid Lanes 4 to 9, *B. subtilis* 168 harboring pHY300-mwp-cotB-ctb-ova epi plasmid (incubation time was 8 h, 10 h, 12 h, 14 h, 16 h, and 18 h, respectively). Bacteria were precipitated by centrifugation for 3 min at 6,800×g. A 15 ul aliquot of precipitated bacteria were used for each lane.

expressed in *B. subtilis* by pHY300-mwp-cotB-ctb-ova epi recombinant expression plasmid. **Figure 3.18** shows that a maximal amount of expressed protein corresponding to the theoretical molecular weight of CotB-CTB-OVA epi (49.6 kDa) was observed at 10 h (**lane 5, Figure 3.18**). From **Figure 3.18**, we also found the expressed antigen protein was degraded with the increase of culture time. This is probably due to proteases produced in *B. subtilis* because proteins with smaller sizes (about 12kDa and 40kDa) assumed as proteolytic products appear at 10 h and later (lane 5 to 9, **Figure 3.18**). The result of western blotting analysis indicated that the CotB-CTB-OVA epi antigen protein was successfully expressed in *B. subtilis*

### 3.3.6.2 Immunofluorescence microscopy

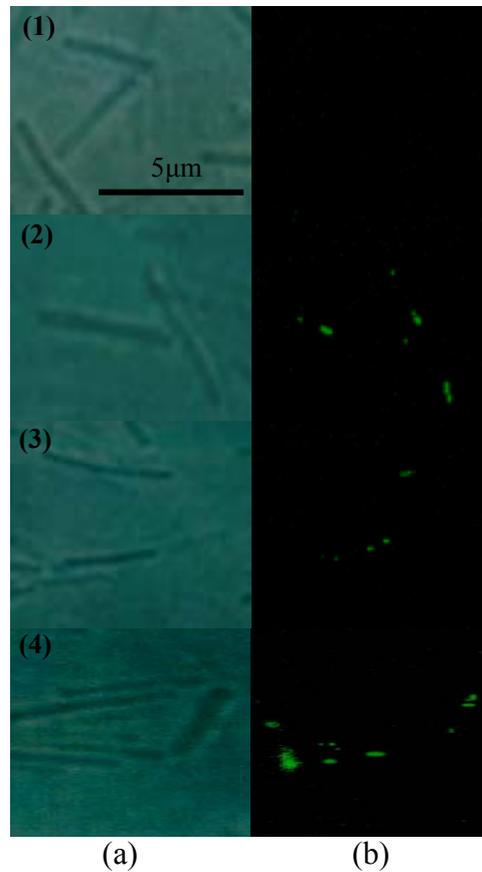


Figure 3.19 Immunofluorescent labeling of transformed *B. subtilis* cells. (1) *B. subtilis* 168 cells harboring the plasmid pHY300-mwp-ctb-ova were grown at 37 °C for 8h in LB medium (negative control). (2), (3), (4) *B. subtilis* 168 cells harboring the plasmid pHY300-mwp-cotB-ctb-ova were grown at 37 °C for 8h in LB medium. Cells were labeled with the primary rabbit anti-cholera toxin antibody, followed by the secondary goat anti-rabbit IgG conjugated with FITC. Bright-field images (a) and the corresponding fluorescence (b) micrographs are shown ( $\times 1500$ ).

Immunofluorescence labeling of cells was performed using rabbit anti-cholera toxin antibody as the primary antibody and goat anti-rabbit IgG conjugated with FITC as the secondary antibody. As shown in **Figure 3.19**, the green fluorescence of the immunostained CTB-OVA fusion protein was observed in *B. subtilis* 168 cells

harboring the plasmid pHY300-mwp-cotB-ctb-ova epi, whereas cells harboring the control plasmid pHY300-mwp-ctb-ova epi were not immunostained. This indicates that CTB-OVA was displayed on the cell surface of *B. subtilis* by CotB anchoring protein.

### 3.3.6.3 Flow cytometric analysis

Flow cytometry was used for the quantitative analysis of the cell surface display of CTB-OVA epi. The *B. subtilis* cells harboring the plasmid pHY300-mwp-cotB-ctb-ova epi and the plasmid pHY300-mwp-ctb-ova epi (negative control without cotB) were stained with the rabbit anti-cholera toxin antibody as the primary antibody and goat anti-rabbit IgG conjugated with FITC as the secondary antibody respectively. As a result, *B. subtilis* cells harboring the plasmid pHY300-mwp-cotB-ctb-ova epi showed a significantly greater intensity of fluorescence signals (**curve 3, Figure 3.20 (b)**) than the control *B. subtilis* cells harboring the plasmid pHY300-mwp-ctb-ova epi (**curve 3, Figure 3.20 (a)**). As shown in **Figure 3.20 (a)** and **(b)**, *B. subtilis* cells harboring the plasmids which were not treated with the primary antibody and the secondary antibody still showed a low fluorescence signals (**curve 1, Figure 3.20 (a)** and **curve 1, Figure 3.20 (b)**). It is possibly due to the fluorescent constituents of *B. subtilis* cells. In addition, even in the presence of the primary antibody, the fluorescence profile of negative control was almost the same as that in the absence of the primary antibody (**curve 3, Figure 3.20 (a)**). Therefore, the fluorescence from negative control was due to the non-specific binding of the FITC-conjugated secondary antibody. This result is consistent with the data shown in **Figure 3.19**. Together with the results in **Figure 3.19**, the results shown here indicate that the CTB-OVA epi antigen was successfully displayed via CotB protein on the cell surface of *B. subtilis*.

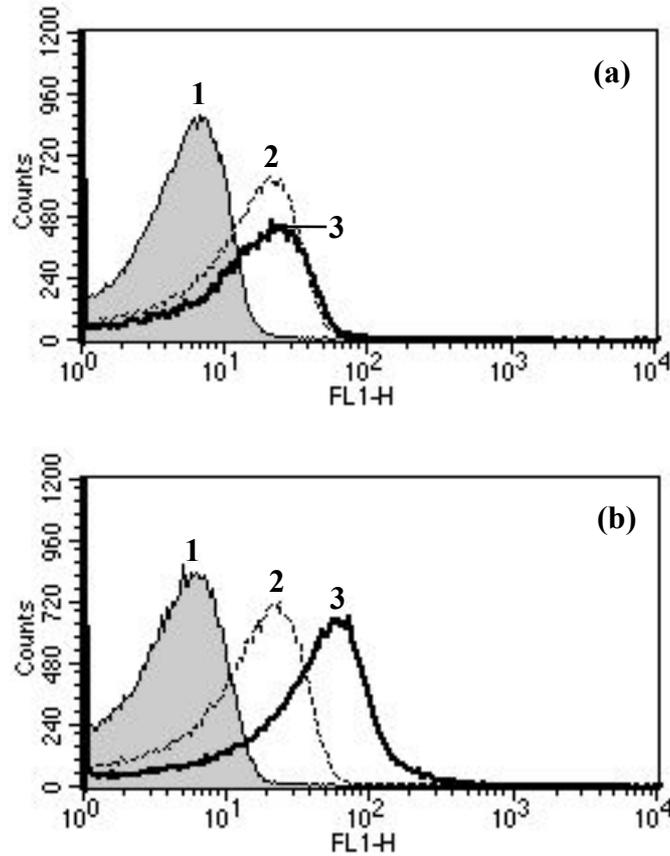


Figure 3.20 Flow cytometric analysis of *B. subtilis* harboring the plasmid.

(a) pHY300-mwp-ctb-ova epi (negative control without cotB) (b) pHY300-mwp-cotB-ctb-ova epi. 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-cholera toxin antibody, followed by the secondary goat anti-rabbit IgG conjugated with FITC. In each experiment, 50,000 cells were analyzed. Curve 1, *B. subtilis* cells harboring the plasmids (negative control without antibody); Curve 2, *B. subtilis* cells harboring the plasmids labeled with the secondary antibody (negative control without the primary antibody); Curve 3, *B. subtilis* cells harboring the plasmids labeled with the primary and secondary antibodies.

### 3.4 Conclusions and Discussions

I report here the use of a novel, CotB-based surface display system to express CTB-OVA epi antigen protein on the surface of vegetative cells of *B. subtilis*. CotB

protein, one of outer coat protein of *B. subtilis* spore, was used as an anchoring motif to express the tetanus toxin fragment C (TTFC) of *Clostridium tetani* on the surface of *B. subtilis* spores [51] by integration of the CotB gene and TTFC gene into the *B. subtilis* genome. The procedure of gene integration seemed complex and time-consuming and the incubation period for sporulation usually required 24h, in addition, the formed spores still needed to be washed and purified [51]. In this study, I considered that CotB can be employed as an anchoring motif to display the antigen protein on the surface of vegetative cells of *B. subtilis* directly so that the procedure of protein expression can be simplified. Furthermore, there is little evidence of expression of heterologous protein on the vegetative cells of *B. subtilis*. *B. subtilis* is non-pathogenic, in addition, it is a bacterium which is closely related to an edible bacterium, *Bacillus subtilis* (natto) used to produce fermented soybean “natto” [10, 11]. Therefore, the development of vaccine delivery systems using *B. subtilis* is significant and promising.

In this study, I constructed a recombinant expression vector based on pHY300 vector by inserting 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 process of surface displayed bacterial vaccine to a great extent. However, the expressed CTB-OVA epi antigen protein on the cell surface of *B. subtilis* were degraded with the increase of culture time probably due to proteases produced in *B. subtilis*. The amount of expressed CTB-OVA epi protein by this surface display system is not very high according to the difference of fluorescence intensity of 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 ordinary system but also establish a good foundation for using *B. subtilis* (natto) as a organism for the production of edible vaccine vehicles in the future research. I described the expression of antigen protein from the gene in the genome of *B. subtilis*. In future experiments, CotB-CTB-OVA epi gene will be integrated into the genome of

*B. subtilis* for the stability of the antigen gene. Further investigation on the increase of the yield and the examination of immunities of displayed protein on the surface of *B. subtilis* cell is still required.

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# Appendix

## Abbreviations used in this dissertation

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Name	Abbr.
<i>Bacillus subtilis</i>	<i>B. subtilis</i>
Cholera Toxin B subunit	CTB
Green Fluorescent Protein	GFP
Allergen from the pollen of <i>Cryptomeria japonica</i>	Cry j
Immunoglobulin E	Ig E
Tetanus Toxin Fragment C	TTFC
Severe Acute Respiratory Syndrome	SARS
<i>Escherichia coli</i>	<i>E. coli</i>
Polymerase Chain Reaction	PCR
Ribosome Binding Site	RBS
Luria-Bertani	LB
Spizizen's Minimal Medium	SMM
Isopropyl- $\beta$ -D-thiogalactopyranoside	IPTG
Phosphate Buffered Saline	PBS
Bovine Serum Albumin	BSA
Polyvinylidene Difluoride	PVDF
Ovalbumin	OVA
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Chloramphenicol	Cm
Ampicillin	Amp
Kanamycin	Kana
Tetracycline	Tc
Blasticidin S	Bs

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## (2) Reagents used in this dissertation

Name of reagent	Composition	
<b>LB medium</b> (pH 7.2)	Tryptone	10g
	Yeast Extract	5g
	NaCl	10g
	Distilled water	to 1000ml
<b>SOC medium</b>	bacto tryptone	20g
	bacto yeast extract	5g
	NaCl	0.5g
	1M MgSO <sub>4</sub>	10ml
	1M MgCl <sub>2</sub>	10ml
	1M Glucose	20ml
	Distilled water	to 1000ml
<b>SMM medium</b>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	<b>0.2%</b>
	K <sub>2</sub> HPO <sub>4</sub>	<b>1.4%</b>
	KH <sub>2</sub> PO <sub>4</sub>	<b>0.6%</b>
	Sodium citrate (2H <sub>2</sub> O)	<b>0.1%</b>
	MgSO <sub>4</sub> · 7H <sub>2</sub> O	<b>0.02%</b>
	Glucose	<b>0.5%</b>
<b>PBS buffer</b> (pH 7.4)	NaCl	8g
	Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	2.9g
	KCl	0.2g
	KH <sub>2</sub> PO <sub>4</sub>	0.2g
	Distilled water	to 1000ml

<b>2×SDS sample buffer</b>	SDS solution (10%)	4ml
	0.5M Tris-HCl buffer (pH 6.8)	2ml
	β-mercaptoethanol	1.2ml
	Glycerol	2ml
	Bromophenol blue solution (1%)	several drops
	Distilled water	to 10 ml
<b>5×electrode buffer</b> (pH 8.3)	Tris	15g
	Glycine	72g
	SDS	5g
	Distilled water	to 1000ml
<b>Blotting buffer</b>	Tris	12g
	Glycine	14.4g
	Methanol	200ml
	Distilled water	to 1000ml