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Comparison of the stability of eGFP displayed on the *Bacillus subtilis* spore surface using CotB and C-terminally truncated CotB proteins as an anchoring motif under extreme conditions

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Abstract

We investigated the expression and stability of enhanced green fluorescent protein (eGFP) under extreme conditions using two types of high-copy-number vectors and two types of anchoring motifs (CotB and C-terminally truncated ΔCotB spore coat proteins) for the development of a spore surface display system in *Bacillus subtilis*. The fused *cotB-gfp* and *ΔcotB-gfp* DNA fragments were cloned into the pUB19 (pUB110-derived) and pHY300PLK vectors. Four types of expression vectors were transformed into *B.* subtilis 168. The expression level of eGFP on the surface of spores prepared from *B. subtilis* transformants was measured by flow cytometry. When pUB19 vector was used, the activities of ΔCotB-eGFP and CotB-eGFP were 17.9 and 5.6 times higher than those of the pHY300PLK vector, respectively. In addition, the activity of pUB19-ΔCotB-eGFP was 1.76 times higher than that of pUB19-CotB-eGFP. Overall, the activity of eGFP was more stable under extreme conditions (heat, pH, and protease challenges) when ΔCotB was used as an anchoring motif instead of CotB. Compared to the control groups, the activities of ΔCotB-eGFP and CotB-eGFP were maintained at 56% and 41% at 80 °C and 88% and 55% at pH 10, respectively. The activities of ΔCotB-eGFP and CotB-eGFP were maintained at 62% and 41%, respectively, when treated with 0.03 U of proteinase K. In addition, the activities were maintained at 77% and 36%, respectively, when treated with 5.5 U of trypsin.

Keywords: Anchoring motif, CotB, eGFP, Spore surface display, Stability

Introduction

Recently, spore surface display studies have been conducted to express proteins or peptides on the spore surface of spore-forming bacteria such as *Bacillus subtilis* [1, 2]. When a target protein of interest is fused to a spore coat protein mainly used as an anchor protein, spore-producing bacteria display the corresponding protein on the spore surface. Depending on the displayed protein, spores can be used as biocatalysts, vaccines, animal probiotics, adsorbents, etc. [3–5]. There are several advantages when using the spores of *B. subtilis* for surface display. *B. subtilis* is generally recognized as a safe organism that has been used as a probiotic in humans and animals and can be easily cultured and stored. In addition, the stability of the target protein expressed on the spore surface can be improved, large-size proteins can be displayed, and proteins toxic to the host cell can be expressed [6].

Bacillus subtilis forms a dormant cell (spore) when exposed to extreme conditions such as malnutrition. The spore is surrounded by a thick peptidoglycan layer with an internal centered chromosome. This layer is called the cortex, and the cortex is surrounded by the inner

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coat, outer coat, and crust, which are formed by various densely stacked coat proteins [7]. This unique structure allows the spore to survive extreme environments such as external heat, dryness, and low pH. In addition, the spore can survive for an infinite period and is able to germinate into a vegetative cell when placed in a conducive environment.

In *B. subtilis*, CotB, CotC, CotG, CotE, CotX, CotY, and CotZ, which constitute spore coat proteins, have been successfully used as anchoring motifs to fuse the target protein for spore surface display [1, 8–11]. The stable and efficient display of the protein on the spore surface can be affected by the anchoring motif used [1]. CotB is the first coat protein used as an anchoring motif and has been used to express several antigens or biocatalysts [8]. CotB is composed of 380 amino acids and has a serine-rich C-terminus (at position 253–380) with strong hydrophilic properties [8]. This C-terminal region contains a high proportion of serine and lysine residues, thereby increasing the possibility of cross-linking between proteins, which may lead to structural instability [7]. The full length of CotB (380 amino acids) as an anchoring motif does not display tetanus toxin fragment C (TTFC) on the spore surface; however ΔCotB (275 amino acids), in which the C-terminal region of CotB is removed, can stably express TTFC [8]. Nevertheless, the full length of the CotB protein has also been used to successfully express the anthrax of *Bacillus anthracis* and alpha toxin of *Clostridium perfringens* [12, 13]. Recently, a number of studies have expressed foreign proteins using ΔCotB as an anchoring motif [14–17]. However, in these studies, the stability of the displayed proteins under extreme conditions (heat, pH and protease challenges) remains unclear. The stability of proteins displayed under harsh conditions is critical for a wide range of industrial applications involving spore surface display technology.

In this study, we used the CotB and ΔCotB (249 amino acids) proteins as an anchoring motif and enhanced green fluorescent protein (eGFP, F64L/S65T) as a target protein to construct a foreign protein expression system using *B. subtilis* spores. GFP is small in size, easy to form a fusion protein, and can be used to monitor gene expression [18]. The expression level of eGFP was compared using two *E. coli–B. subtilis* shuttle vectors for high level expression. In addition, to investigate the stability of eGFP displayed on the spore surface under extreme conditions, the activity of eGFP was measured after treatment with heat, acidic or alkaline solutions, and proteases.

Materials and methods

Bacterial strains, culture condition, and transformation

Escherichia coli DH5α and *Bacillus subtilis* 168 strains were used for cloning experiments. Both strains were cultured in Luria–Bertani (LB) medium at 37 °C. The transformation of *E. coli* DH5α was carried out using an electroporator (Gene Pulser II; Bio-Rad, Hercules, CA, USA) under the following conditions; 25 μF capacitance, 200 Ω resistance, and 2.5 kV/cm. The transformation of *B. subtilis* 168 was performed as described previously [19]. Ampicillin (50 μg/mL), kanamycin (10 μg/mL) and tetracycline (10 μg/mL) were used to screen *E. coli* or *B. subtilis* transformants.

Plasmid construction

The primers and plasmids used in this study are shown in Table 1. The *E. coli–B. subtilis* shuttle vector pHY300PLK was purchased from Takara Bio (Otsu, Japan). The construction scheme for the recombinant plasmids is shown in Fig. 1.

The high-copy-number *E. coli–B. subtilis* shuttle vector pUB19 was constructed by the fusion of the pUC19 and pUB110 plasmids. After the cleavage of both plasmid DNAs with *Eco*RI and *Bam*HI, the 2.6 kb fragment of pUC19 and 3.8 kb fragment of pUB110 were ligated to construct pUB19 plasmid (6.4 kb).

The *cotB* gene encoding coat protein B (CotB) and the *ΔcotB* gene encoding C-terminally truncated coat protein B (ΔCotB) were amplified from the chromosomal DNA of *B. subtilis* 168 by PCR. A 1.3 kb DNA fragment containing the promoter and structural gene sequence of *cotB* was amplified by the forward primer COTB-F and reverse primer CBGP-R. A 0.9 kb *ΔcotB* DNA fragment was amplified with the COTB-F and COP-R primers.

The *gfp* gene encoding eGFP was obtained from the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA) by PCR amplification. The *gfp-1* DNA to be fused with *cotB* DNA was amplified using the CBGP-F and GFP-R primers. The *gfp-2* DNA to be fused with *ΔcotB* DNA was amplified using the COP-F and GFP-R primers. The amplified *cotB* DNAs and *gfp* DNAs were recombined by overlap extension PCR [20] using the COTB-F and GFP-R primers. As a result, 2 kb *cotB-gfp-1* and 1.8 kb *ΔcotB-gfp-2* DNA fragments were obtained. Since the structural gene sequences of *gfp-1* and *gfp-2* are identical, they are only referred to as *gfp* in the rest of the text. The recombined *cotB-gfp* DNA and *ΔcotB-gfp* DNA were ligated into the pTOP-V2 vector (Enzynomics, Daejeon, Korea) to construct pTOP-*cotB-gfp* (6 kb) and pTOP-*ΔcotB-gfp* (5.6 kb).

In the final step, *cotB-gfp* and *ΔcotB-gfp* DNA fragments were subcloned into the *E. coli–B. subtilis* shuttle vectors pUB19 and pHY300PLK. The pUB19-*cotB-gfp* (8.7 kb) plasmid was constructed by ligation of the 2.3 kb *Bam*HI-*Xho*I fragment of pTOP-*cotB-gfp* and the 6.4 kb *Bam*HI–*Sal*I fragment of pUB19. The pUB19-*ΔcotB-gfp* (8.2 kb) plasmid was constructed by ligation of the 1.8 kb

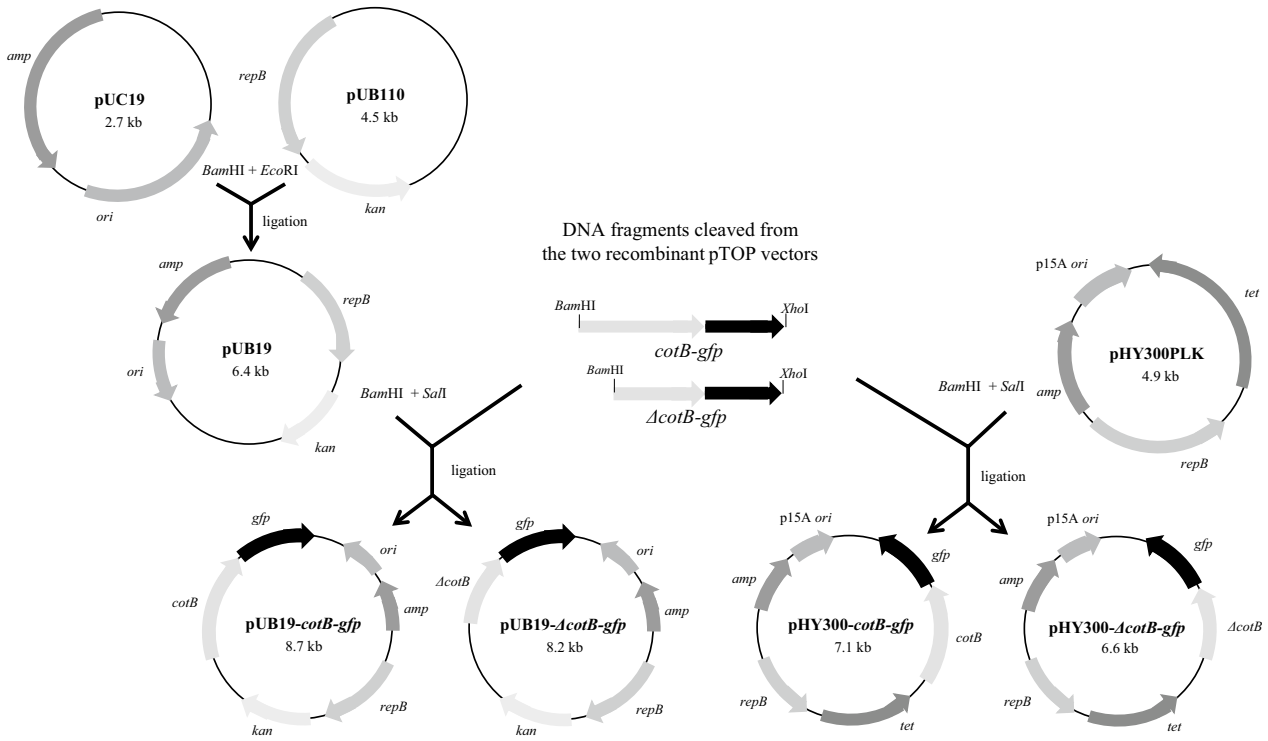

Fig. 1 Construction scheme for the recombinant plasmids

*Bam*HI-*Xho*I fragment of pTOP-*ΔcotB-gfp* and the 6.4 kb *Bam*HI–*Sal*I fragment of pUB19. The pHY300-*cotB-gfp* (7.1 kb) plasmid was constructed by ligation of the 2.3 kb *Hin*dIII–*Xho*I fragment of pTOP-*cotB-gfp* and the 4.8 kb *Hin*dIII–*Sal*I fragment of pHY300PLK. The pHY300-*ΔcotB-gfp* (6.6 kb) plasmid was constructed by ligation of the 1.8 kb *Eco*RI fragment of pTOP-*ΔcotB-gfp* and the 4.8 kb *Eco*RI fragment of pHY300PLK.

Preparation of *B. subtilis* spores

The transformants of *B. subtilis* 168 containing the pUB19-*cotB-gfp*, pUB19-*ΔcotB-gfp*, pHY300-*cotB-gfp*, or pHY300-*ΔcotB-gfp* plasmids were cultured in Difco sporulation medium. Spores were obtained from each culture according to the Nicholson and Setlow method [21]. The spores were resuspended in sterile phosphate-buffered saline (PBS, pH 7.4) and stored at − 20 °C. The colony-forming unit (cfu) of the spores was determined by serial dilution plating on a LB agar plate containing the appropriate antibiotics.

Measurement of eGFP fluorescence under extreme conditions

To investigate the stability of eGFP on the spore surface under extreme conditions, the spore suspension was treated with heat, acidic or alkaline solutions, and proteases, and the residual activity of eGFP was measured by flow cytometry. Spores were washed three times with sterile distilled water before use, and the final concentration of the spores in the suspension was adjusted to 1×10^5 cfu/mL.

For the thermal stability analysis of eGFP at different temperatures (40, 50, 60, 70, 80, 90, and 100 °C), 0.1 mL of spores and 0.1 mL of sterilized water were mixed in an Eppendorf tube and heated for 10 min in a heating block. The control group was analyzed at room temperature (25 °C). For the pH stability analysis of eGFP, Mcilvaine's buffer (pH 4, 5, 6, 7, and 8) and glycine–NaOH buffer (pH 9 and 10) were used. The spores (0.1 mL) and 0.1 mL of each buffer solution were mixed and incubated at 37 °C in a water bath for 1 h. The control group was analyzed at pH 8. For proteolytic resistance analysis of eGFP, 0.1 mL of spores were mixed with 0.1 mL of trypsin solution (5500, 550, 55, and 5.5 units in PBS buffer) or 0.1 mL of proteinase K solution (3, 0.3, 0.03, and 0.003 units in PBS buffer) and incubated at 37 °C for 1 h. In both cases, the control group was not treated with the enzymes. After each treatment, the spores were washed three times with PBS buffer, and the mean fluorescence intensity (MFI) of eGFP was measured. The MFI of each control groups was normalized to 100.

Confocal microscopy and flow cytometry analysis

The fluorescence images of the spores were obtained using a confocal microscope (Nikon Eclipse Ti; Nikon, Tokyo, Japan). The excitation filter was 488 nm and the photographs were taken using the confocal microscope.

The fluorescence of the spores displaying eGFP was measured using the Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) equipped with a 488-nm argon laser as the excitation light source. Spore samples were injected into the flow cytometer and the fluorescence in the range of 518–548 nm was measured with a fluorescence detector. Flow cytometry data were analyzed using BD Accuri C6 software. The experiment was carried out in triplicate.

Statistical analysis

All data presented in this study are expressed as mean ± standard deviation from triplicate samples. The statistical significance between groups was determined with an unpaired two-tailed t-test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) at a significance level of $p < 0.05$.

Results and discussion

Construction of recombinant plasmids

pHY300PLK is known as a high-copy-number *E. coli–B. subtilis* shuttle vector used for studying the expression of the gene of interest [22, 23]. pUB110 is a broad-host-range plasmid originating from *Staphylococcus aureus* and can be replicated in *B. subtilis* at a copy number of 50–100 per cell [24]. pUB110-derived plasmids have also been used for high-level production of various recombinant proteins in *B. subtilis* [25, 26]. In this study, the *E. coli–B. subtilis* shuttle vector pUB19 was constructed using the pUB110 and pUC19 plasmids to express eGFP (Fig. 1).

In order to select a more suitable vector for the surface display of eGFP on the *B. subtilis* spore, the fused *cotB-gfp* and *ΔcotB-gfp* genes were cloned into the two shuttle vectors pUB19 and pHY300PLK (Table 1). Finally, four types of vectors (pUB19-*cotB-gfp*, pUB19-*ΔcotB-gfp*, pHY300-*cotB-gfp*, and pHY300-*ΔcotB-gfp*) were constructed and introduced into *B. subtilis* 168 to investigate the expression of eGFP and its stability under extreme conditions.

Display of eGFP on the *B. subtilis* spore surface

Several studies have used the CotB and ΔCotB proteins as an anchor protein to display various passenger proteins on the spore surface [12, 13, 15, 16]. However, comparative studies of the expression level of passenger proteins and their stability under extreme conditions using either anchor protein are limited. In this study, ΔCotB (249 amino acids) was used as an anchor protein, in which the serine-rich C-terminal region is removed. Confocal microscopy and flow cytometry were used to

Table 1 Primers and plasmids used in this study

	Description	Source or reference
Primers		
CotB-F	CGACGCGTGTATATTAAAAACCGTTCAC	This study
CBGP-R	GCTCACCATATGATAGTCTATCGTTCTGACAATCG	This study
CoP-R	CTCGCCCTTGCTCACCATATGATTGCTGTCCTTATCATTATTGTCTTC	This study
CBGP-F	ATAGACTATCATATGGTGAGCAAGGGCGAG	This study
GFP-R	ACTAGCTAGCCATTATAAGCTGCA	This study
CoP-F	GAAGACAATAATGATAAGGACAGCAATCATATGGTGAGCAAGGGCGAG	This study
Plasmids		
pUC19	*E. coli* vector, Ap^r	Lab collection
pUB110	*B. subtilis* vector, Km^r	Lab collection
pEGFP-N1	Eukaryotic expression vector carrying *gfp*	Clontech
pTOP-V2	*E. coli* cloning vector, Ap^r, Km^r	Enzynomics
pHY300PLK	*E. coli-B. subtilis* shuttle vector, Ap^r, Tc^r	Takara Bio. Co., Ltd.
pUB19	*E. coli-B. subtilis* shuttle vector, Ap^r, Km^r	This study
pTOP-V2-*cotB-gfp*	cloning of *cotB-gfp* fusion gene	This study
pTOP-V2-*ΔcotB-gfp*	Cloning of *ΔcotB-gfp* fusion gene	This study
pUB19-*cotB-gfp*	Spore display of GFP using CotB anchor	This study
pUB19-*ΔcotB-gfp*	Spore display of GFP using ΔCotB anchor	This study
pHY300-*cotB-gfp*	Spore display of GFP using CotB anchor	This study
pHY300-*ΔcotB-gfp*	Spore display of GFP using ΔCotB anchor	This study

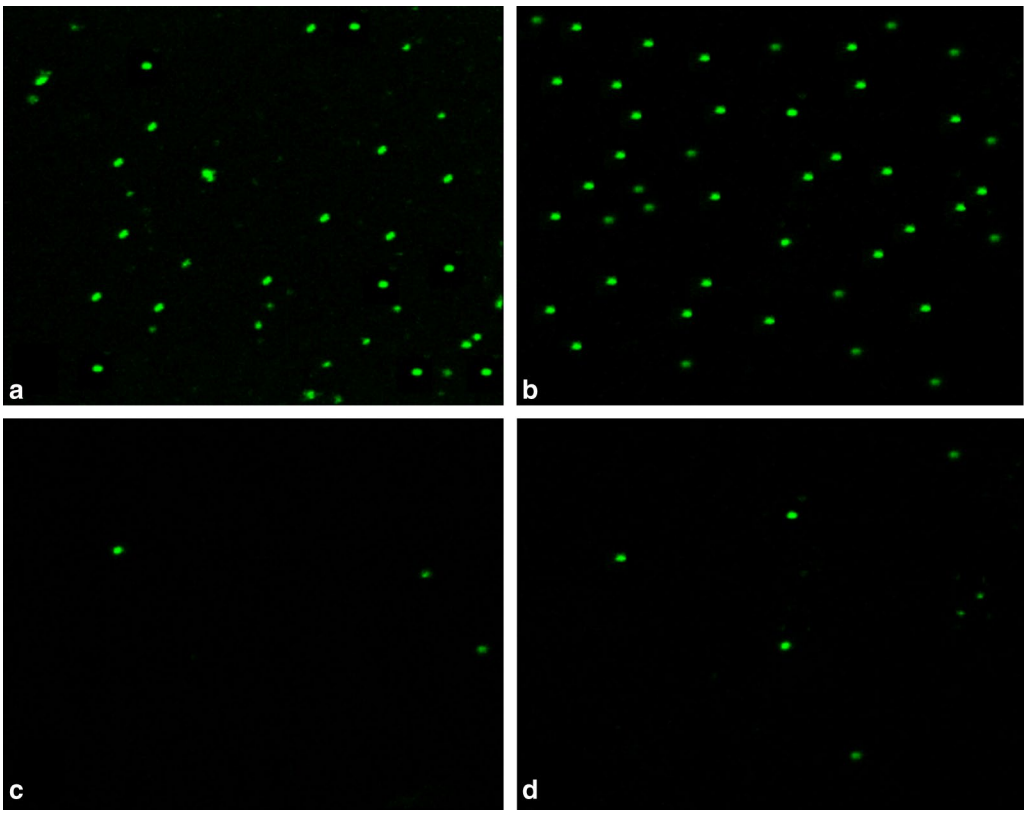

Fig. 2 Detection of eGFP expression on the surface of spores prepared from *B. subtilis* 168 harboring the plasmids pUB19-*cotB-gfp* (**a**), pUB19-*ΔcotB-gfp* (**b**), pHY300-*cotB-gfp* (**c**), and pHY300-*ΔcotB-gfp* (**d**)

confirm the expression of eGFP on the surface of spores prepared from *B. subtilis* 168 transformants harboring pUB19-*cotB-gfp*, pUB19-*ΔcotB-gfp*, pHY300-*cotB-gfp*, and pHY300-*ΔcotB-gfp* plasmids. Confocal microscopy revealed that the fluorescence of eGFP was well expressed for all four types of spores (Fig. 2). Although the results are qualitative, the number of spores exhibiting fluorescence was higher when using the pUB19 plasmid compared with the pHY300PLK plasmid. Therefore, to quantitatively compare the fluorescence of each spore groups, it is necessary to measure their fluorescence intensity.

The MFI of each spore group was measured at room temperature by flow cytometry to compare the fluorescence intensity between spores (Fig. 3). When eGFP was expressed by the pUB19 plasmid, the MFIs of ΔCotB-eGFP and CotB-eGFP spores were 12,244 and 6971, respectively. The activity of ΔCotB-eGFP was around 1.76 times higher than that of CotB-eGFP. The use of ΔCotB lacking the C-terminal region as an anchor protein of eGFP was more preferable than the use of CotB. This result was in good agreement with the results of other studies that expressed foreign proteins using CotB with the C-terminal end removed, which may be associated with structural instability [8, 14]. On the other hand, when eGFP was expressed using the pHY300PLK plasmid, the MFIs of ΔCotB-eGFP and CotB-eGFP were 684 and 1238, respectively. Comparing these values

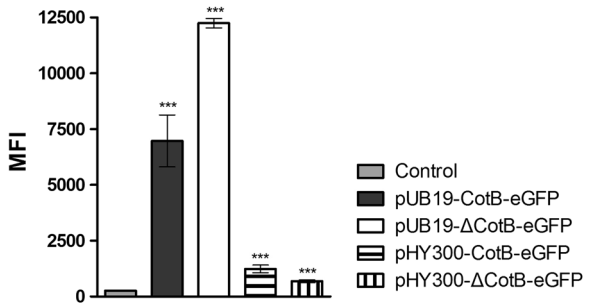

Fig. 3 Mean fluorescence intensity (MFI) of eGFP-displaying spores prepared from *B. subtilis* 168 harboring the plasmids pUB19-*ΔcotB-gfp*, pUB19-*cotB-gfp*, pHY300-*cotB-gfp*, and pHY300-*ΔcotB-gfp*. *B. subtilis* 168 without a plasmid was used as a negative control. Statistical significance between the groups is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

with those of the pUB19 vector, the activities of ΔCotB-eGFP and CotB-eGFP were 17.9 and 5.6 times lower, respectively.

In a previous study, when *N*-acetyl-D-neuraminic acid aldolase was displayed on the *B. subtilis* spore surface, the use of a high-copy-number vector was more beneficial than a low-copy-number vector for *N*-acetyl-D-neuraminic acid production [10]. In order to express and display the CotB fusion proteins on the *B. subtilis* spore surface, the endogenous wild-type CotB proteins are required [8]. This dependence implies that competition between the wild-type CotB proteins and the CotB fusion proteins may occur during the assembly of coat proteins [27]. In a previous study using CotB protein as an anchoring motif, the spores contained much more wild-type CotB protein than CotB fusion protein [8]. Therefore, in this study, the use of a high-copy-number vector seems desirable, as the fused coat proteins (ΔCotB-eGFP and CotB-eGFP) must compete with the wild-type CotB coat proteins during the assembly of spore coat proteins. Although we did not measure the copy number of the plasmids used, the use of the pUB19 plasmid was more suitable than the pHY300PLK plasmid for the display of eGFP on the *B. subtilis* spore. Therefore, in the following experiments, eGFP expressed by the pHY300PLK plasmid was excluded because of the low fluorescence intensity.

Thermal stability of eGFP on the spore surface

Various proteins have been successfully displayed on the *B. subtilis* spore surface using several anchor proteins; however, their stability under extreme conditions is unclear. To investigate the thermal stability of the displayed eGFP on the spores, the activities of ΔCotB-eGFP and CotB-eGFP expressed by the pUB19 plasmid were measured after heat treatment (40–100 °C) (Fig. 4). The activity of ΔCotB-eGFP and CotB-eGFP began to gradually decrease at temperatures above 50 °C. At 70 °C, the residual activities of ΔCotB-eGFP and CotB-eGFP were 81% and 62%, respectively, compared with the activity of the control measured at 25 °C. The activities of ΔCotB-eGFP and CotB-eGFP were maintained at 56% and 41%, respectively, at 80 °C. However, nearly all activity was lost above 90 °C. Overall, the activity of ΔCotB-eGFP was more stable than that of CotB-eGFP at temperatures above 50 °C.

Mutational analysis of *B. subtilis* lipase has revealed that thermostable mutant enzymes have a higher apparent melting temperature and free energy of unfolding compared with wild-type enzymes [28]. Immobilized enzymes tend to increase heat stability and pH tolerance by increasing the structural rigidity of the protein [29]. Similar to immobilized enzymes, eGFP displayed on the spores also showed improved heat stability in this study. In a study in which a fragment of tetanus toxin was displayed using full-length CotB as an anchoring motif, the expression of the fused toxin was not stable due to the serine-rich sequence (248–356 amino acids) in the C-terminal region of CotB [8]. Similarly, our results indicated that the thermal stability of eGFP at high temperatures may be affected by the anchoring motif used.

pH tolerance of eGFP on the spore surface

The fluorescence of wild-type GFP is pH-dependent and stable at pH 6–10, but begins to decrease gradually at pH 6 or lower [30]. In a previous study, the activity of an eGFP (F64L/S65T) mutant was reduced by half at pH 5.5 [31], whereas another GFP mutant (T203I) maintained 90% activity at pH 6 [32].

To investigate the pH tolerance of the displayed eGFP, the spores were treated with various pH buffers (pH 4–10) at 37 °C for 1 h, and the residual activity of eGFP was measured (Fig. 5). As the optimum pH of wild-type

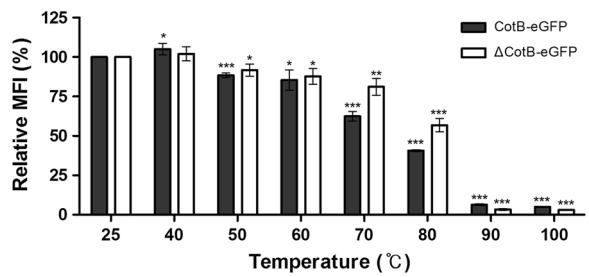

Fig. 4 Relative mean fluorescence intensity (MFI) of eGFP-displaying spores after heat treatment. Spores were prepared from *B. subtilis* 168 harboring the plasmids pUB19-*cotB-gfp* and pUB19-*ΔcotB-gfp*. The MFI at 25 °C was normalized to 100. Statistical significance between the groups is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

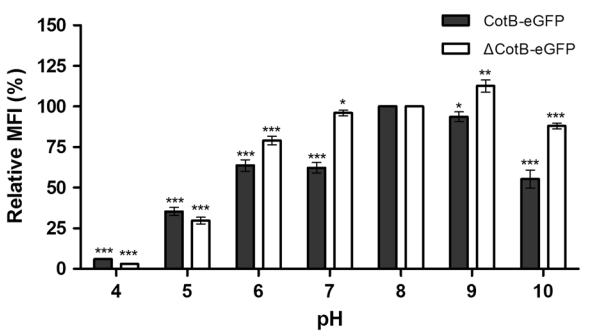

Fig. 5 Relative mean fluorescence intensity (MFI) of eGFP-displaying spores after pH challenge. Spores were prepared from *B. subtilis* 168 harboring the plasmids pUB19-*cotB-gfp* and pUB19-*ΔcotB-gfp*. The MFI at pH 8 was normalized to 100. Statistical significance between the groups is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

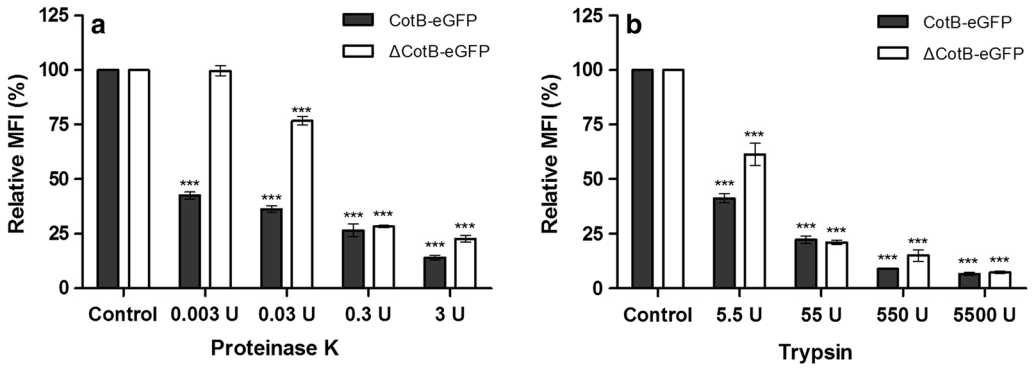

Fig. 6 Relative mean fluorescence intensity (MFI) of eGFP-displaying spores after proteinase K (**a**) and trypsin (**b**) challenges. Spores were prepared from *B. subtilis* 168 harboring the plasmids pUB19-*cotB-gfp* and pUB19-*ΔcotB-gfp*. The MFI without enzyme treatment was normalized to 100. Statistical significance between the groups is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

GFP was 8 [33], the pH of each control group was set to 8. In comparison with the activity of ΔCotB-eGFP at pH 8, the activity of ΔCotB-eGFP was maintained above 96% at pH 7, and the maximum activity was 113% at pH 9. On the other hand, the activity of CotB-eGFP was the highest at pH 8 and was maintained at 62% and 94% at pH 7 and pH 9, respectively. At below pH 5, the activities of both ΔCotB-eGFP and CotB-eGFP were decreased rapidly. Overall, the pH tolerance of ΔCotB-eGFP was better than that of CotB-eGFP in the pH range of 6–10. Therefore, the pH tolerance of fused eGFP may be affected by the presence of an unstable serine repeating structure in the C-terminal region of the anchor protein CotB.

Proteolytic resistance of eGFP on the spore surface

Depending on the proteins displayed, their resistance to proteolytic enzymes may affect industrial applications such as feed additives or edible vaccines. To investigate the proteolytic resistance of the displayed eGFP, the spores were treated with proteinase K and trypsin and the residual activity of eGFP was measured (Fig. 6). When the spores were treated with 0.003, 0.03 and 0.3 units of proteinase K, the activity of ΔCotB-eGFP was maintained at 100%, 77%, and 28%, respectively; in contrast, the activity of CotB-eGFP remained at 42%, 36%, and 26%, respectively, under the same conditions. In addition, when the spores were treated with 5.5 and 55 units of trypsin, the activity of ΔCotB-eGFP was maintained at 62% and 21%, respectively; under the same conditions, the activity of CotB-eGFP was maintained at 41% and 22%, respectively. Although the displayed eGFP was sensitive to protease treatment, the use of ΔCotB as an anchor protein had a greater effect than the use of CotB on protection from proteolytic activity. As mentioned previously, mutant proteins with increased thermostability could exhibit protease resistance due to their structural stability [28]. Similarly, the increased proteolytic resistance of ΔCotB-eGFP appears to be associated with its increased thermal stability.

In conclusion, eGFP was successfully displayed on the *B. subtilis* spore surface by using CotB and C-terminally truncated ΔCotB as anchor proteins. The pUB110-derived plasmid was more suitable than the pHY300PLK plasmid as a vector for the high expression of fused eGFP. A comparison of the thermal stability, pH stability, and proteolytic resistance of eGFP on the spore surface revealed that the activity of eGFP was higher and more stable when ΔCotB was used as an anchoring motif. Although eGFP was evaluated as a passenger protein in this study, our findings would be applicable to the display of other proteins that require stability under extreme conditions.

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Authors' contributions

SJK, EAP, and DHL carried out the experiment. SJK wrote the manuscript with support from EAP and DHL. KWH supervised this work. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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