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Bacterial Surface Display of GFP_{UV} on *Bacillus subtilis* Spores

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Abstract To analyze a *cotG*-based *Bacillus subtilis* spore display system directly, GFP_{UV} was expressed on the surface of *Bacillus subtilis* spores. When GFP_{UV} was fused to the C-terminal of the *cotG* structural gene and expressed, the existence of a CotG-GFP_{UV} fusion protein on the *B. subtilis* spore was confirmed by flow cytometry confocal microscopic analysis. When the *cotG* anchoring motif was deleted, no fluorescence emission was observed under flow cytometry and confocal microscopic analysis from the purified spore, confirming the essential role of CotG as an anchoring motif. This GFP_{UV} displaying spore might be used for another signaling application triggered by intracellular or extracellular stimuli.

Keywords: *Bacillus subtilis*, *cotG*, GFP_{UV}, spore, surface display

Bacterial surface display is based on expressing recombinant proteins fused to sorting signals that direct their incorporation on the cell surface. It has been used in combinatorial polypeptide libraries screening [1], whole cell bioconversion [8], live vaccine production [5], bioremediation [16], and whole cell metal adsorbents [20].

GFP from jelly fish *Aequorea victoria* or its variant GFP_{UV} [4] emit fluorescence when excited by UV or blue light, due to auto catalytic formation of chromophore from the residues Ser-Tyr-Gly. Therefore, they have been widely used for live cell imaging, and gene expression localization mainly because of their small size, easy expression, and no need for another cofactor for functional expression. Additionally, they have been used for the monitoring and estimation of anchoring motif efficiency such as Omp-C [3], InaK [14], Lpp-OmpA [18], vesicular stomatitis virus G glycoprotein [2, 15], or signaling with intracellular or extracellular stimuli [11, 13, 19, 21] in bacteria or phage display without the aid of specific antibody.

Recently, we displayed tetrameric streptavidin of *Streptomyces avidinii* [12] on the surface of *B. subtilis* spores using a spore coat protein, CotG, as an anchoring motif. CotG is a small coat protein transcribed by mother-cell-specific sigma factors (σ^K) [17], and is needed for the assembly of the outer coat layer in the course of *B. subtilis* spore formation.

In this paper, we tried to express GFP_{UV} on the surface of *B. subtilis* spores using a *cotG* anchoring motif to characterize the *cotG* anchoring motif and spore display system more precisely without the aid of specific antibody or other components.

To this end, two kinds of GFP_{UV} expression systems were constructed. One was a soluble GFP_{UV} expression, not fused to the *cotG* structural gene, but intended to be expressed in the cytosol fraction of mother cell (Fig. 1B), and the other was a CotG-GFP_{UV} fusion protein expression for the surface localization, driven by its own *cotG* promoter (Fig. 1C) to synchronize their expression.

Strains, vectors, transformation method, culture conditions, spore purification method, flow cytometry analysis, and confocal analysis were described in the previous paper [7, 9, 12, 14]. Chromosome of *B. subtilis* DB104 [9] and plasmid pGFP_{UV} (Clontech, Palo Alto, CA, U.S.A.) were used for *cotG* and GFP_{UV} cloning as a PCR template. For the construction of CotG-GFP_{UV} fusion protein expression vector, the PCR product containing the *cotG* promoter and whole structural gene obtained with two primers (*cotG* 5': 5'-GCCTTTGGATCCAGTGTCCCTAGCTCCGAG-3' and *cotG* 3'-linker: 5'-CTATTGCTGCAGTGAACCCCCACC-TCCTTTGTATTTCTTTTTGACTA-3') was inserted into pCSK1 with BamHI and PstI digestion. A *cotG* 3'-linker was designed to insert a flexible linker (Gly-Gly-Gly-Gly-Ser) [6] between CotG and GFP_{UV}. Then, DNA fragment containing the GFP_{UV} structural gene and stop codon, obtained with two

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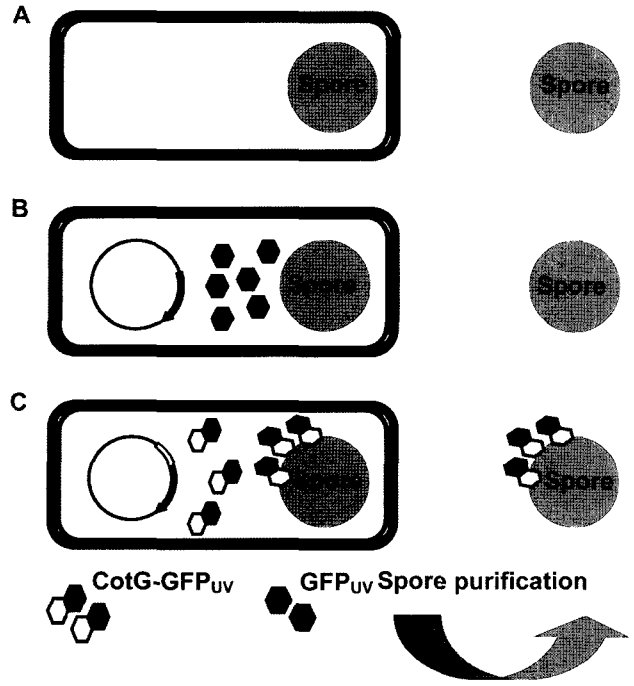

Fig. 1. Schematic illustration of GFP_{UV} expression, before (left) and after (right) spore purification.
(**A**) Host strain DB104 (**B**) Soluble form of GFP_{UV} expression from BJH168 (**C**) CotG-GFP_{UV} fusion expression from BJH167.

primers (*GFP_{UV}* 5': 5'-CCGGTACTGCAGATGAGTAAA-GGAGAAGAACT-3', and *GFP_{UV}* 3': 5'-GAAAAGTGCC-ACCTGACGTC-3') was inserted at the C-terminal of the CotG structural gene with PstI and EcoRI digestion. For the soluble, intracellular expression of GFP_{UV}, the PCR product obtained with two primers (*cotG* 5' and *$cotG_{promoter}$* 3': 5'-ATGGGACTGCAGGCCCAATTTGAAATCCTCCT-3'), containing only *cotG* promoter and start codon (TTG), was inserted into pCSK 1 with BamHI and PstI digestion. The same GFP_{UV} fragment was fused to the start codon (TTG) of the *cotG* promoter with PstI and EcoRI digestion.

In the case of *B. subtilis* DB104 (pSDJH-*$cotG_{Promoter}$-GFP_{UV}*), which was designated as BJH168, purified spore would be deprived of all the GFP_{UV} in the course of spore formation and purification because of the lack of the anchoring motif, CotG (Fig. 1B). Therefore, the fluorescence intensity of the purified spore of BJH168 would be similar to that of DB104, whereas purified spore of *B. subtilis* DB104 (pSDJH-*cotG-GFP_{UV}*), which was designated as BJH167, may have attached CotG-GFP_{UV} fusion (Fig. 1C) and would emit fluorescence. Bacterial cells of BJH168 and BJH167 are expected to emit fluorescence due to the intracellular CotG-GFP_{UV} and soluble GFP_{UV} (Figs. 1B–1C).

For the flow cytometry analysis, DB104, BJH168, and BJH167 were harvested at 16 h after inoculation. The fluorescence intensity of the culture harvest and purified spore of DB104 did not change at all (Fig. 2A). In the case of BJH168, which lacks the *cotG* structural gene, an interesting flow cytometry pattern was obtained (Fig. 2B). When total bacterial culture harvest, which contained vegetative cell, developing spore, and fully developed spore, was examined under flow cytometry, a complicated histogram was observed (Fig. 2B, right peak). Almost all the population showed enhanced fluorescence compared with DB104. However, the flow cytometry pattern looked like a combination of two major fractions. on the right, the small fraction is interpreted as vegetative cell and developing spore, which contains soluble GFP_{UV} in the mother cell fraction, and thereby results in increased fluorescence. On the left, the large fraction is interpreted as fully developed spore, which cannot accumulate GFP_{UV}, and emits no fluorescence. A

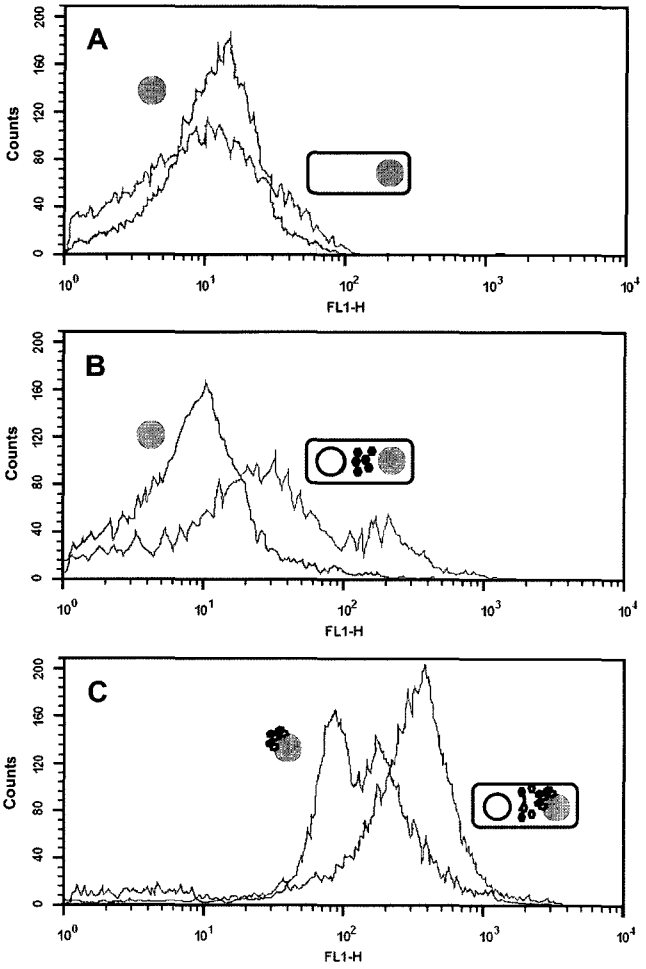

Fig. 2. Flow cytometry of whole bacterial cell and purified spore expressing different types of GFP_{UV}.
A. DB104; **B**. Soluble form of GFP_{UV} expression from BJH168; **C**. CotG-GFP_{UV} fusion expression from BJH167. In each panel, flow cytometry from whole bacterial cell (right) and from purified spore (left) is presented separately. Developing spore in the mother cell of *B. subtilis* and purified spore are represented as grey solid circles.

small increase in fluorescence compared with DB104 can be interpreted as nonspecific binding of GFP_{UV} to the spore surface. After spores had been completely purified from the culture harvest, no change in the fluorescence intensity was observed (Fig. 2B, left peak). This phenomenon is exactly what was expected. Owing to the lack of an anchoring motif, the soluble form of GFP_{UV} was washed out in the course of spore purification.

In the case of BJH167, which carries a *cotG* anchoring motif, the fluorescence intensity of the culture harvest was highest and uniform (Fig. 2C, right peak). Its spore also gave high fluorescence compared with that of DB104 or *cotG*-lacking BJH168 (Fig. 2C, left peak). These results indicate that the CotG-GFP_{UV} fusion is expressed as an active fusion protein in the case of BJH167, and the fusion protein is embedded in the spore coat. The difference of fluorescence between culture harvest and purified spore corresponds to the total amount of expressed CotG-GFP_{UV} fusion and the amount of CotG-GFP_{UV} fusion incorporated into the spore surface. Taking into account of the log scale of the x-axis, a considerable amount of expressed CotG-GFP_{UV} fusion protein was lost in the course of spore formation and purification. The remaining CotG-GFP_{UV} fusion will be incorporated into the outer coat layer of the developing spore. If we consider that there is one more intact copy of *cotG* in the *B. subtilis* chromosome, and fusion protein CotG-GFP_{UV} is competing with the original coat protein, CotG, then this reduction of fluorescence after spore purification can be explained. Overall, those results once again clearly show that CotG is essential for the proper incorporation of fusion protein (CotG-GFP_{UV}) into the developing spore coat of *B. subtilis*.

To observe surface-expressed GFP_{UV} directly, DB104 (Fig. 3A) and GFP_{UV}-expressing BJH167 (Figs. 3B–3C) were prepared, and the culture harvest (Figs. 3A–3B) or purified spore (Fig. 3C) was observed under laser confocal microscopy. No fluorescence was observed from DB104 (Fig. 3A). In the case of the culture harvest of BJH167, which expresses the CotG-GFP_{UV} fusion protein (Fig. 3B), fluorescence from the developing spore can be observed. Fluorescence from two different fractions, one from CotG-GFP_{UV} fusion incorporated in the developing spore and the other from CotG-GFP_{UV} fusion released in the cytosol of *B. subtilis*, contributed to the rod-shaped fluorescence image of the GFP_{UV} expressing pattern (Fig. 3B). Spore of BJH167 gave round-shaped fluorescence emission (Fig. 3C), due to the complete removal of CotG-GFP_{UV} fusion suspended in the cytosol of *B. subtilis*. Laser confocal analysis again confirmed the surface localization of CotG-GFP_{UV} fusion protein on the surface of *B. subtilis* spore, and the essential role of *cotG* for this display of target protein.

In this experiment, our designed expression of GFP_{UV} with or without the *cotG* structural gene demonstrated clearly that *cotG* serves as an efficient anchoring motif in the *B. subtilis* spore display system. More than 50 coat proteins are known to be involved in *B. subtilis* spore coat assembly. GFP_{UV} fused to other coat proteins, can be an efficient and rapid tool for the identification of possible coat protein. Furthermore, the essential binding motif or domain can be localized by the display of a partial coat protein-GFP_{UV} fusion. Recently, massive coat protein-GFP fusion or GFP expression by a sporulation-related promoter was used for the identification of a spore coat protein interaction network [10]. Furthermore, this GFP_{UV}-displaying spore might be used for another signaling application triggered by intracellular or extracellular stimuli and for environmental monitoring of toxic compounds.

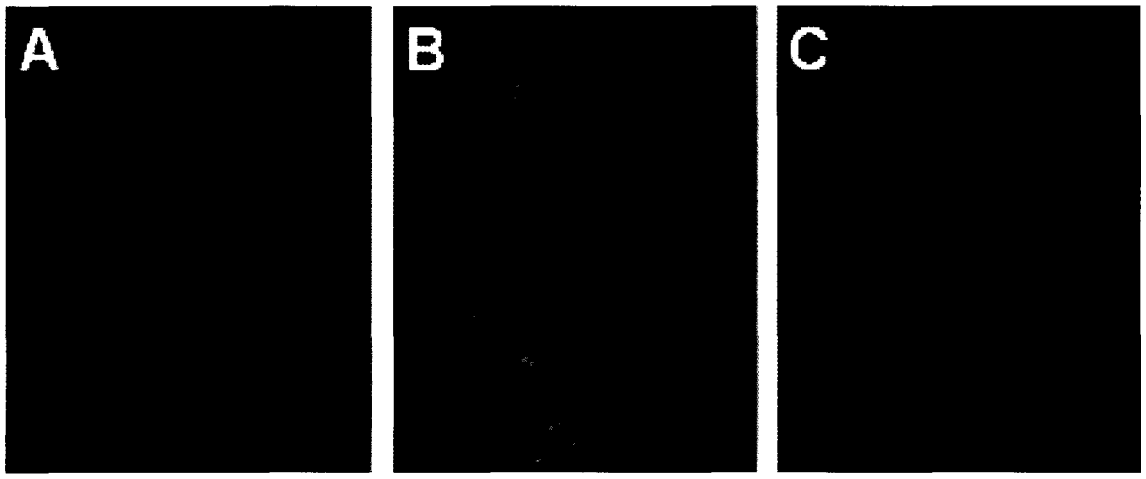

Fig. 3. Laser confocal microscopic analysis.
A. Culture broth of DB104; **B**. Culture broth of BJH167; **C**. Purified spore of BJH167.

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