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Spore-displayed streptavidin: A live diagnostic tool in biotechnology

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Abstract

Streptavidin, which is one of the most widely used proteins in biotechnological application field and is active only in tetrameric form, was surface expressed on the surface of *Bacillus subtilis* spore. Spore coat protein of *B. subtilis*, CotG, was used as an anchoring motif to display streptavidin. FACS using anti-streptavidin antibody was used for the verification of surface localization of expressed CotG-streptavidin fusion protein. FACS and dot-blot were used for the verification of biological activity of displayed streptavidin with FITC-labeled biotin.

Keywords: Bacterial surfce display; Streptavidin; *Bacillus subtilis*; Spore

Streptavidin, which is a 60 kDa protein produced by *Streptomyces avidinii*, binds to biotin very tightly with a K_d value of 10^{-15}. Active streptavidin is composed of four molecules of streptavidin monomer with a molecular weight of 15 kDa and four active sites for biotin binding exist in the active tetrameric streptavidin. Biotin can be easily conjugated to various biomolecules such as proteins, nucleic acids, and carbohydrates, thereby enabling sensitive detection by streptavidin.

Since its first cloning by Cantor group [1], many papers were published on the expression and production in various hosts. It can be produced from its original host, *S. avidinii* [2,3]. Gram-positive strain *Bacillus subtilis* was also employed for the expression [4–6]. In *Escherichia coli*, it was also produced intracellularly as a soluble [7] or insoluble [8] form. In spite of the above-mentioned cloning and expression in various hosts, there still exist some kinds of difficulties in streptavidin expression and utilization. One of the initial problems is its high G + C content of encoding sequence (69%), which makes its cloning very difficult at its PCR amplification step. Even to solve this problem, Thompson and Weber reported synthetic streptavidin gene, which changed all the possible DNA sequences without affecting acid sequences considering codon usage of *E. coli* [5,9]. Another serious problem in active streptavidin expression is the sequestration of intracellular biotin by the expressed streptavidin, which is lethal to host bacteria. To alleviate this problem, elimination of basal level gene expression by tightly regulated inducible promoter [9] or synthesis of higher level of intracellular biotin was tried [6], or supplementation of biotin in the culture medium was tried. One more remaining problem is the purification of expressed streptavidin. Because, expressed streptavidin is charged with intracellular biotin, folding and refolding of expressed streptavidin or column purification step is generally adopted [6].

Recently, we developed a new display system based on *B. subtilis* spore, which can display a multimeric protein such as β-galactosidase of *E. coli*. [10]. Surface display, including phage display and newly developed spore display, can expose the target protein on the surface of host bacteria, which is a very suitable form of application, where binding or interaction with other molecules is of great importance. The application field of surface display

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covers screening of the target molecules of improved properties such as in vitro evolved enzyme itself [11], enzymes or proteins involved in whole cell bioconversion [12], bioremediation [13], whole cell adsorption [14], and live vaccine production [15]. For these purposes, various surface display systems have been developed in gram-negative bacteria (*lamB*, *phoE* and *ompA*), gram-positive bacteria (*cwbA* and M6), and yeast (*aga1p* and *cwp2p*). But, these conventional surface display systems could not be applied for the display of multimeric enzymes due to their general transport mechanism of target protein across cell membrane and cell wall.

Spore of *B. subtilis* is formed through continuous addition of various coat proteins expressed in the mother cell, thereby eliminating transport steps which block multimer display. Furthermore, spore formation is the late stage of cell growth, when no cell division occurs. It is expected that lethality arising from biotin sequestration may not affect vitality and integrity of spore. One more expected advantage is the easy purification steps, because spore can be easily separated from vegetative cell simply by centrifugation. The aim of this study was the surface display of active tetrameric streptavidin on the surface of *B. subtilis* spore without affecting the viability of host cell and easy preparation of streptavidin coated spore for the biotin-related application.

Materials and methods

Bacterial strains, plasmids, and culture conditions. Two protease-deficient strain, i.e., *B. subtilis* DB104, was used as a host strain for the surface display of the streptavidin on *Bacillus* spore. GYS medium comprising $(NH_4)_2SO_4$ 2 g, yeast extract 2 g, K_2HPO_4 3.3 g/L, sodium citrate 0.1 g, and glucose 0.1 g/L supplemented with trace elements of $MnSO_4$ H_2O 0.1 g, $CaCl_2$ 0.16 g, and $MgSO_4 7$ H_2O 0.82 g/L was used for bacterial cell culture, and the culture was maintained at 37 °C in a shaking incubator. Chloramphenicol (5 μg/ml) was added into the medium for the selection of *B. subtilis* harboring pSDJH-*cotG-sav*.

Vector and strain construction. pCSK1, derived from pHPS9, which is low copy, *E. coli*/*B. subtilis* shuttle vector, was used for the stable expression of fusion protein in *B. subtilis*. pHPS9 was digested with *Eco*RI and *Hin*dIII, and Klenow enzyme was used to fill both sides of the vector. p123T (EMBL database Accession No. Z46733, Mobitec) was digested with *Bss*HII, and the resulting small fragment, which contains the multi-cloning site of p123T, was ligated into the Klenow-treated DNA fragment of pHPS9. This *E. coli*/*B. subtilis* shuttle vector, containing the multi-cloning site of p123T, was named as pCSK1. PCR product, containing cotG promoter and structural gene, was obtained with two primers (CotG-5′: GCC TTT GGA TCC AGT GTC CCT AGC TCC GAG, CotG-3′: CTA TTG CTG CAG TGA ACC CCC ACC TCC TTT GTA TTT CTT TTT GAC TA) using *Bacillus* chromosome as PCR template. PCR product was digested with *Bam*HI and *Pst*I, and ligated into same enzyme digested pCSK1 to yield pSDJH-cotG. Primer CotG-3′ was designed to have a flexible linker (Gly-Gly-Gly-Gly-Ser) at the C-terminal of the cotG structural gene, which is fused to the N-terminal of streptavidin. The resulting plasmid was named as pSDJH100. Two primers (sav-*Pst*I-5: AGCG CTCTGCAGGACCCCTCCAAGGACTCGAAG, sav-*Eco*RI-3: AT GCATGAATTCGAGGTCCCGGCACCCGCCGG) were used for the cloning of streptavidin with plasmid pSUC2, which contains full sequences of streptavidin, as a template. Those primers were designed to contain the mature gene of streptavidin, removing the signal sequence of streptavidin. PCR product and pSDJH100 were digested with *Pst*I and *Eco*RI, and ligated to yield pSDJH-*cotG-sav*, which expresses CotG-Linker (GGGGS)-Streptavidin fusion driven by its own cotG promoter. The two-step (SP I, SP II) method was used for the transformation of pSDJH-*cotG-sav* into *B. subtilis* DB104 and chloramphenicol (5 μg/ml) was used for the selection.

Spore purification method. Cells were cultivated for 16–20 h at 37 °C with 200 rpm. Vegetative cells, sporulating cells, and spores were harvested and purified using either lysozyme treatment or renografin (sodium diatrizoate, S 4506, Sigma) gradient method [10]. Purified spores were confirmed under microscope.

FACS analysis. For immunofluorescence staining, purified spores were washed three times with PBS solution. The washed spores were resuspended in 1 ml PBS solution containing anti-streptavidin antibody (1:500) (S 6390, Sigma) for 1 h on ice. After the spores were washed three times with PBS again, the spores were incubated with FITC-labeled secondary antibody for 1 h on ice, subsequently washed three times with PBS, and resuspended in 500 μl PBS. For streptavidin activity analysis, the prepared spore was incubated with biotin-FITC conjugated and washed three times with PBS again. These spores were examined under a flow cytometer (FACstar/plus, Becton–Dickinson, Oxnard, CA). Software CellQuest ver. 1.0 was used for data analysis.

Analysis of fluorescent image. Fluorescent images were observed in confocal fluorescence microscopy with an FITC filter (LSM5 Pascal, Carl Zeiss, Germany). The fluorescent intensities were analyzed by Zeiss image software. Samples were scanned using the 488 nm wavelength from an argon laser. Because the fluorescence has an emission peak in the frequency corresponding to the green color, the only green component of the image was analyzed.

Results

Construction of CotG-streptavidin fusion expression vector

Streptavidin is a small protein of 183 amino acids, and its N-terminal 24 amino acids are removed when it is secreted into culture medium. Primers were designed that anchoring motif (CotG) and linker (GGGGS) are directly connected to the full-length streptavidin of processed form (from 25th residue (Asp) to the end). The constructed expression vector is presented (Fig. 1). Original host *cotG* promoters were used for the expression of fusion protein to synchronize spore formation and incorporation of expressed fusion proteins into the spore.

Verification of surface display of CotG-streptavidin fusion protein

The expression of streptavidin on the external surface of *B. subtilis* spore was verified by fluorescence-activated cell sorter (FACS) (Fig. 2). Purified spores of DB104 and DB104 (pSDJH-*cotG-sav*) were treated with anti-streptavidin antibody and FITC-labeled secondary antibody. Washed spore was examined under a flow cytometer. Spore of DB104 (pSDJH-*cotG-sav*) showed significantly improved fluorescence intensity compared

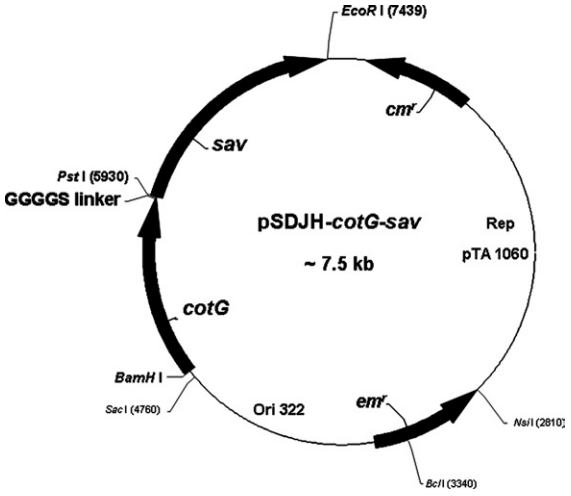

Fig. 1. Construction of CotG-streptavidin expression vector. Between anchoring motif and streptavidin, flexible linker peptide sequence for Gly-Gly-Gly-Gly-Ser was inserted. Size of the vector and positions of restriction site are the approximate value.

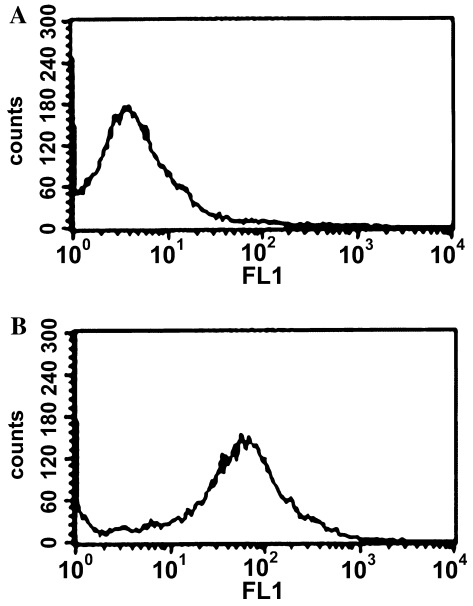

Fig. 2. FACS histograms of purified spores of (A) DB104 and (B) DB104 (pSDJH-*cotG-sav*) to identify surface localization of expressed CotG-Sav. fusion with anti-sav antibody and FITC-labeled secondary antibody.

to the control spore of DB104. This means that *cotG* serves as an efficient anchoring motif as in the previous case [10], and CotG-streptavidin fusion protein is in a position where anti-streptavidin antibody can easily access.

Biotin binding of surface-expressed CotG-streptavidin fusion protein

In the previous section, we confirmed the location of CotG-streptavidin fusion using anti-streptavidin antibody. In many papers dealing with streptavidin expression, expressed streptavidin is charged with intracellular biotin, which requires the purification of expressed streptavidin. If spore-displayed CotG-streptavidin fusion is completely charged with intracellular biotin, it needs another step of purification. To observe the biotin binding activity of surface expressed streptavidin, dot-blot experiment was done (Fig. 3). Purified spores of DB104 and DB104 (pSDJH-*cotG-sav*) were dot-blotted on slide glass using a dot-spotting apparatus. The size of each dot is about 6 mm each. FITC-biotin was sprayed on it, and after 5 min, the slide glass was gently washed with PBS 5 times. It was observed with a confocal fluorescence microscope. On panel (A), where the control spore of DB104 was dot-blotted and processed, only the background level of fluorescence was observed. On panel (B), distinctive fluorescence was observed. Fluorescence was not uniform in the whole area of dot. Strong fluorescence was observed at the outermost circle of each dot. This phenomenon is interpreted as resulting from the configuration of dot-blotting needle or fluid-dynamic nature in the small area of dot with 6 mm of diameter coupled with the hydrophobicity of *B. subtilis* spore. But, this result clearly demonstrates that CotG-streptavidin fusion expressed on spore surface binds to biotin labeled with FITC. Though we do not know how much fraction of expressed CotG-streptavidin fusion protein is charged with intracellular biotin, spore-expressing CotG-streptavidin fusion can be directly used for biotin-related application.

One more method for the confirmation of activity of expressed CotG-streptavidin fusion protein is by directly using FACS. We are using FITC-biotin conjugates, if CotG-streptavidin fusion expressing spore can bind to FITC-biotin, it can be detected by FACS (Fig. 4) like when we used anti-streptavidin antibody for the determination of surface localized CotG-streptavidin fusion protein. Purified spores of DB104 and DB104 (pSDJH-*cotG-sav*) were prepared, incubated with FITC-biotin for 10 min, washed with PBS 3 times, and

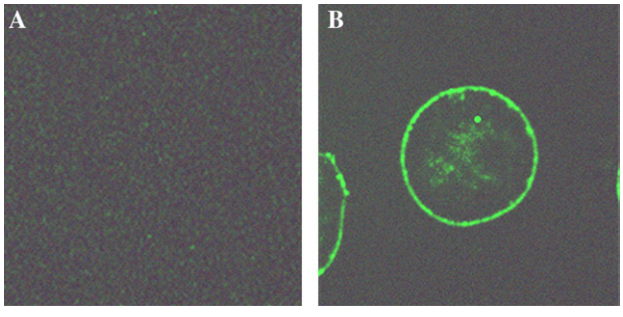

Fig. 3. Fluorescence microscopy of purified spores of (A) DB104 and (B) DB104 (pSDJH-*cotG-sav*) which were dot-blotted on slide glass showing streptavidin–biotin binding. The size of each dot is around 6 mm.

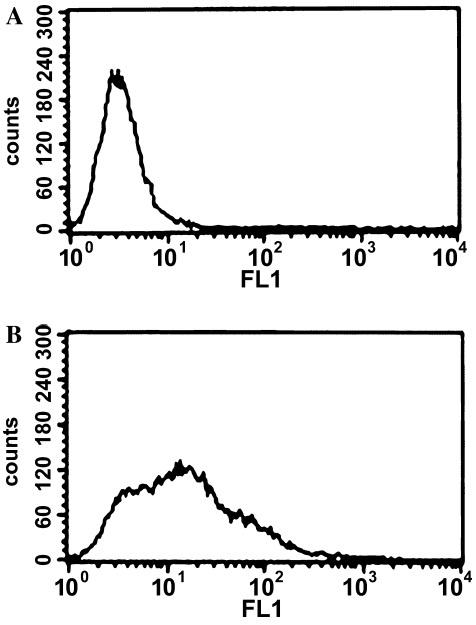

Fig. 4. FACS histograms of purified spores of (A) DB104 and (B) DB104 (pSDJH-*cotG-sav*) showing the activity of surface-displayed streptavidin directly to identify surface localization of expressed CotG-Sav. fusion with anti-sav antibody and FITC-labeled secondary antibody.

observed under a flow cytometer. We can see the difference in fluorescence between the control spore of DB104 (Fig. 4A) and the CotG-streptavidin expressing spore of DB104 (pSDJH-*cotG-sav*) (Fig. 4B).

Protease accessibility test

Protease treatment of spore gives evidence for the surface localization of target proteins, for exogenously added protease cannot penetrate through the spore wall. Purified spores of DB104 (pSDJH-*cotG-sav*) were suspended in PBS buffers containing each 0.1% protease for 1 h. A control sample with the spores was also made in PBS buffer without protease. After protease treatment and washing, the spore was incubated with FITC-biotin for 10 min, washed with PBS 3 times, and observed under flow a cytometer. When CotG-streptavidin expressing spore was treated with Trypsin (Sigma T 8642) proteinase from *Aspergillus oryzae* (Sigma P 4032), protease from *Bacillus licheniformis* (Sigma, P 6670), we can see the reduced fluorescence (Figs. 5C–E) compared to the intact spore of DB104 (pSDJH-*cotG-sav*) (Fig. 5B).

Discussion

In our recent publication [10], we suggested a new method of surface display system based on *B. subtilis* spore. Various anchoring motifs were developed for the display of tetrameric β-galactosidase of *E. coli*. This spore-based display system was also effective for enzyme

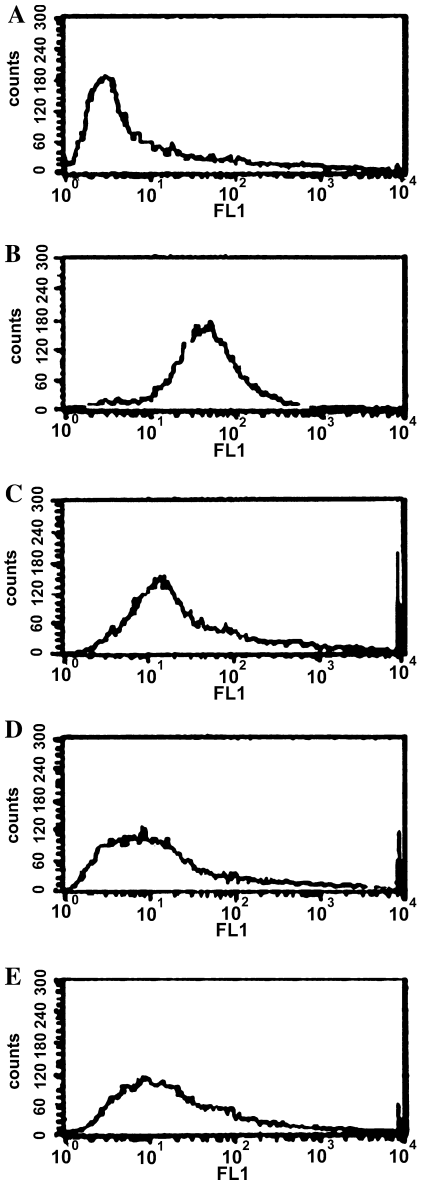

Fig. 5. FACS histograms of purified spores of (A) DB104 and (B) DB104 (pSDJH-*cotG-sav*) with protease treatment. Spore of DB104 (pSDJH-*cotG-sav*) was treated with (C) trypsin, (D) proteinase from *Aspergillus oryzae* (Sigma P-4032), and (E) protease from *Bacillus licheniformis* (Sigma, P-6670). After protease treatment, each spore was incubated with biotin-FITC, washed with PBS, and observed with FACS.

reaction containing organic solvent in the case of β-galactosidase [10] or lipase [16]. In this paper, we tried another important application of tetrameric streptavidin display using *cotG* anchoring motif. All the data presented in this paper, FACS analysis using anti-streptavidin antibody, dot-blot analysis with FITC-biotin, and protease accessibility test, prove the first successful surface display of tetrameric streptavidin, which was not possible with the other bacterial surface display system. Furthermore, spore display system overcame the toxicity caused by sequestration of intracellular biotin, and

surface-displayed CotG-streptavidin fusion was functionally active without further purification.

There were some reports of displaying multimeric protein in the field of phage display. Using a variant of phage P8 coat protein, streptavidin was displayed [17]. β-Galactosidase of *E. coli* was also displayed using the major tail protein of bacteriophage λ [18,19]. It can be understandable if we think that phage assembly and spore formation share a common mechanism in that coat protein of phage of spore is synthesized in the cytosol of the mother cell, and after the lysis of the mother cell, phage or spore is liberated to culture medium. With this common mechanism spore display has one more advantage over phage display in that displayed enzyme can be directly screened through FACS technology, using fluorescently labeled antibodies. The size of phage particle limits its compatibility with current FACS technology [9].

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