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Displaying a recombinant protein on flocs self-produced by
Escherichia coli through fused expression with elongation factor
Ts

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Highlights

We demonstrated expression of a fusion protein consisting of Tsf and GFP on *E. coli* flocs.

The percentage of Tsf-GFP in total floc protein reached approximately 15% (w/w).

This technique could display heterologous protein on the *E. coli* floc structure.

1 **ABSTRACT**

2 The utility of engineering flocculation is wildly recognized in applied and
3 environmental microbiology. We previously reported self-produced flocculation of
4 *Escherichia coli* cells by overexpressing the native *bcsB* gene that encodes a component
5 of the cellulose synthesis pathway. Further experiments clarified that the spontaneous *E.*
6 *coli* flocs were proteinous, and elongation factor Ts (Tsf) was the main component. In
7 this study, we demonstrated successful expression of a fusion protein consisting of Tsf
8 and green fluorescence protein (GFP) on *E. coli* flocs. Interestingly, the percentage of
9 Tsf-GFP in total floc protein reached approximately 15% (w/w). The proposed design of
10 a fusion protein with Tsf enables displaying a recombinant target protein on the floc
11 structure.

INTRODUCTION

Flocculation is an aggregation phenomenon of bacterial cells in the form of flocs or flakes. Numerous microorganisms have been found to show floc-forming capabilities [1] that can be applied to wastewater treatment. In activated sludge, the components of flocs typically include polysaccharides, polynucleotides, and proteins [2].

Escherichia coli is a typical laboratory microbe that lacks the floc-forming ability. Many researches have attempted to induce floc formation of *E. coli* by adding artificial flocculants consisting of inorganic compounds including aluminum and cationic polymers such as chitosan [3-5]. We have previously reported self-produced flocculation of *E. coli* cells by overexpressing the native *bcsB* gene [6] that encodes a component of transmembrane cellulose synthase [7]. Further experiments clarified the spontaneous *E. coli* flocs were proteinous, and elongation factor Ts (Tsf) had the highest score among floc proteins in nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [8]. Tsf is known to promote the release of GDP by forming an intermediate complex with another elongation factor, Tu, which is involved in the elongation cycle of protein biosynthesis [9]. These results promoted us to evaluate Tsf for displaying a heterologous protein on the proteinous structure of self-produced *E. coli* flocs.

In this study, we designed a fusion protein consisting of Tsf and green fluorescence protein (GFP), which was expressed during the process of *E. coli* floc formation. Observations of GFP revealed localization of Tsf protein within the floc structure. Furthermore, quantitative evaluation based on western blotting was conducted to determine the amount of fusion protein throughout the floc structure.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* K-12 strain BW25113 was obtained from the National BioResource Project (National Institute of Genetics (NIG), Mishima, Japan) [10]. The pNTR-SD-*bcsB* plasmid [11] was provided by NIG and the transformant was named BW25113/*bcsB* that forms spontaneous flocs [8]. pCA24N and ASKA-*tsf-gfp* plasmids were also provided by NIG [12]. To express GFP, the pCA24N-*gfp* plasmid was constructed from pCA24N by adjusting the *gfp* gene in frame [12]. BW25113/*bcsB* cells were transformed with pCA24N-*gfp* and ASKA-*tsf-gfp* plasmids and the resultant strains were named BW25113/*bcsB/gfp* and BW25113/*bcsB/tsf-gfp*, respectively.

E. coli cells were cultured in lysogeny broth (LB) medium (10 g/L Hipolypeptone, 5 g/L Bacto-yeast extract and 10 g/L NaCl). The culture medium for strains harboring the plasmids contained with 50 mg/L ampicillin or 25 mg/L chloramphenicol. All test cultures were precultured in LB medium for 14 h at 37°C and then inoculated into L-tubes containing fresh LB medium with 2 g/L glucose (LB-G) giving an optical density of 660 nm (OD_{660}) = 0.01.

Flocculation of each *E. coli* strain

For floc formation, *E. coli* cells were cultured in 10 mL LB-G medium at 37°C in an L-tube with shaking at 45 strokes/min. The recombinant strains were cultured in medium containing isopropyl β -D-1-thiogalactopyranoside (1 mM), ampicillin (50 mg/L) and chloramphenicol (25 mg/L). After 24 h of culture, the L-tubes containing the floc and cell suspension were left to stand at room temperature for 15 min and the

resultant precipitates were resuspended in a 0.9% (w/v) NaCl solution. The resultant flocs were transferred into glass bottles and photographed using a digital camera under irradiation by fluorescent light or black light (peak wavelength; 365 nm) lamp to confirm GFP expression. For quantitative assays, the resultant flocs were washed four times with the 0.9% (w/v) NaCl solution to remove loosely adsorbed cells on the flocs. The amount of protein in the flocs was measured by a PierceTM BCA protein Assay Kit (Thermo Fisher Scientific Inc., UK) and the value was used as an index of floc amount as described previously [13]. The number of *E. coli* cells included in the floc structure was estimated by measuring ATP as described elsewhere [13]. ATP measurement was conducted using an ATP Colorimetric/Fluorometric Assay Kit (BioVision Inc., Milpitas, CA) according to the manufacturer's procedure.

Microscopic observation of flocs

Flocs were observed under an inverted Eclipse Ti microscope with NIS-Elements Advanced Research software (Nikon Corp., Japan). Prior to analysis, the flocs were fixed with 2.5% (v/v) glutaraldehyde for 1 h. After washing, DNA of *E. coli* cells in the floc structure was stained with 20 mg/L propidium iodide (PI) by incubation for 30 min in the dark. The samples were observed under sequential excitation at wavelengths of 488 and 561 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analyses of flocs

A floc sample (5 μ L) was subjected to SDS-PAGE. For western blotting, proteins were transferred from the gel to a membrane sheet of Hybond P (GE Healthcare Ltd., UK)

1 using the semi-dry transfer method. Hybridization was conducted using an anti-GFP
2 primary antibody (Medical & Biological Laboratories Co., Japan) and an ECL Western
3 Blotting Starter Kit (GE Healthcare Ltd.) according to the manufacturers' protocols.
4 Hybridization signals were detected using a ChemiDoc imaging system (Bio-Rad
5 Laboratories Inc., Hercules, CA).

6 The protein bands were analyzed by densitometry (Image J software; NIH, Bethesda,
7 MD) as an index of target protein expression. The amounts of GFP and Tsf-GFP were
8 determined using purified GFP and Tsf-GFP as standards. Purification of GFP and
9 Tsf-GFP was conducted using a Bio-scale Mini Profinity IMAC cartridge and Profinia
10 protein purification system (Bio-Rad Laboratories Inc.) after ultrasonication of *E. coli*
11 cells expressing each protein. Finally, the percentages of GFP and Tsf-GFP were
12 determined based on total floc protein determined by the BCA method.

14 **RESULTS AND DISCUSSION**

15 **Flocculation of *E. coli* cells**

16 In our previous study, we confirmed floc formation of *E. coli* BW25113/*bcsB* cells
17 [8]. In this study, we also observed the flocculation of *E. coli* BW25113/*bcsB/gfp* and
18 BW25113/*bcsB/tsf-gfp* cells (Fig. 1A). The flocs formed by these cells were
19 approximately 1–2 mm and similar to those formed by BW25113/*bcsB* cells.
20 Observations under black light irradiation showed that the flocs of BW25113/*bcsB/gfp*
21 and BW25113/*bcsB/tsf-gfp* cells produced green fluorescence (Fig. 1A), suggesting that
22 the flocs included GFP within their structure. In particular, the intensity of green
23 fluorescence in flocs formed by BW25113/*bcsB/tsf-gfp* cells was stronger than that in
24 flocs formed by BW25113/*bcsB/gfp* cells.

Next, the amount of protein in flocs was determined by the BCA method. In our previous study, it was confirmed that the main components of *E. coli* flocs were proteinous, and determination of the protein concentration could be used as an index for the amount of flocs [13]. Compared with BW25113/*bcsB* cells (320 mg/L), the amounts of floc proteins from BW25113/*bcsB/gfp* and BW25113/*bcsB/tsf-gfp* cells were 260 and 270 mg/L, respectively. There was no significant adverse effect of GFP or Tsf-GFP expression on the floc formation of BW25113/*bcsB* cells (Fig. 1B). Furthermore, the number of cells per unit weight of floc protein was estimated by the ATP content and compared among each strain. As shown in Fig. 1C, the numbers of BW25113/*bcsB*, BW25113/*bcsB/gfp*, and BW25113/*bcsB/tsf-gfp* cells included in the flocs were about 1.2×10^8 , 1.2×10^8 and 1.4×10^8 cells/mg-floc protein, respectively, suggesting that expression of GFP or Tsf-GFP did not affect the cell density within the floc structure. Thus, GFP and Tsf-GFP were successfully expressed without a negative effect on the properties of flocs, such as formation efficiency and cell inclusion. As for the reason why the expression of Tsf-GFP did not affect the properties of flocs, it is thought that the native Tsf protein contributes to maintaining the properties of flocs. In this study, we used the wild type strain as a host instead of *tsf*-deficient mutant strain since *tsf* is known as an essential gene in *E. coli* cells [14]. Therefore, BW25113/*bcsB/tsf-gfp* cells express both native Tsf and recombinant Tsf-GFP. It can be speculated that the expression of native Tsf assists oligomerization of recombinant Tsf-GFP into the self-produced flocs while keeping the state of cells and properties of flocs.

Observation of flocs

Figure 2 shows images of typical flocs formed by respective *E. coli* strains. In the case of the BW25113/*bcsB* strain, optical microscopy showed fibrous structures on the surfaces of flocs (see bright field image in Fig. 2). Additionally, three-dimensional images obtained by confocal scanning laser microscopy (CLSM) revealed that PI-labeled *E. coli* cells were dispersed within the floc structure. Considering that the flocs were washed several times with saline solution before observation, it can be speculated that the *E. coli* cells are firmly bound to the flocs. These results also support our previous study indicating that flocs mainly contain polymer fibers rather than cell aggregates [8]. As expected, flocs formed by BW25113/*bcsB* cells did not show any green fluorescence because of the lack of GFP introduction (Fig. 2A). In the case of flocs formed by BW25113/*bcsB/gfp* cells (Fig. 2B), green fluorescence was scattered in spots within the floc structure. The spots of PI-labeled *E. coli* cells were overlapped with green fluorescence, suggesting that GFP was expressed within the cytoplasm of individual *E. coli* cells. In the case of BW25113/*bcsB/tsf-gfp* cells (Fig. 2C), the PI-labeled *E. coli* cells were also dispersed within the floc structure similarly to the cases of the other two strains. However, it is worth noting that green fluorescence was observed throughout the whole floc structure, even in spaces without PI-labeled cells. These observations support our strategy of displaying Tsf-GFP on the floc structure for secretion from *E. coli* cells, as expected based on our previous results showing that the floc component was largely Tsf protein.

Quantitative evaluation of GFP and Tsf-GFP within the floc structure

To determine the amounts of GFP and Tsf-GFP within the floc structure, the proteins in flocs were analyzed by western blotting. For comparison, the flocs were separated

from suspended cells by sedimentation and each fraction was analyzed by western blotting. The fraction of flocs still included cells trapped inside the floc structure. As shown in Fig. 3A, in the case of BW25113/*bcsB/gfp* cells, calibration analysis using purified GFP produced a clear single band at around 29 kDa, as predicted by the molecular weight of GFP plus the additional sequence, and showed load dependency (Fig. 3A). Samples from both flocs and cells also showed the band at around 29 kDa. There was no significant difference in the amount of GFP between flocs and cells. Conversely, in the case of the BW25113/*bcsB/tsf-gfp* strain, the bands in the calibration analysis of purified Tsf-GFP appeared at around 75 kDa that was larger than expected molecular weight of the fusion protein (60 kDa). The cell sample produced a single weak band at 75 kDa, whereas bands of the floc sample appeared at around both 60 and 75 kDa. This split of the band could not be improved by experimental modifications. It can be speculated that some Tsf-GFP molecules in the calibration samples and cell culture samples might not be denatured completely by SDS-treatment and appeared at larger size in the electrophoresis.

Next, GFP and Tsf-GFP were quantitatively analyzed by densitometry of the bands. In the case of Tsf-GFP in flocs, the band split at both 60 and 75 kDa. Therefore, two types of data were calculated. One only estimated the band at 75 kDa, and the other estimated both bands as the whole quantity. Fig. 3B shows the percentage of target protein in total cell proteins or total floc proteins. In the case of BW25113/*bcsB/gfp* strain, the percentage of GFP in cells and flocs were $2.2 \pm 0.6\%$ (w/w) and $2.5 \pm 0.9\%$ (w/w), respectively. There was no significant difference. In contrast, as for BW25113/*bcsB/tsf-gfp* strain, whereas the percentage of Tsf-GFP in cells was $0.6 \pm 0.4\%$ (w/w), the values in flocs with and without including the band at 60 kDa were

1 14.8±7.3% (w/w) and 7.6±3.2% (w/w), respectively. Considering that the number of
2 cells per unit weight of floc proteins was not significantly different in these flocs (Fig.
3 1C), the increase in the percentage was not due to the increase of cells within the floc
4 structure. Therefore, these results strongly support the view that Tsf-GFP was
5 transferred from *E. coli* cells to the whole floc structure.

6 We showed that the Tsf-GFP fusion protein was expressed throughout the whole floc
7 structure. In our previous report, Tsf protein had the highest score in NanoLC-MS/MS
8 analysis of floc proteins. Tsf is an elongation factor that promotes the release of GDP by
9 forming an intermediate complex with another elongation factor, Tu, which is involved
10 in the elongation cycle of protein biosynthesis [9]. Tsf has been also identified as an
11 aggregation-resistant protein by *E. coli* proteome-wide analysis in response to a protein
12 denaturant [15]. The synthesis of soluble Tsf was significantly increased even under a
13 strong protein-denaturing environment induced by guanidine hydrochloride. Compared
14 with direct expression, various heterologous proteins are solubly expressed in *E. coli*
15 when subjected to N-terminus fusions with Tsf [15-17]. Thus, Tsf appears to play
16 critical roles in not only the elongation cycle of protein biosynthesis, but also
17 sequencing interactive surfaces of heterologous proteins from nonspecific
18 protein-protein interactions leading to the formation of inclusion bodies. Even though
19 there is a gap between these reported functions of Tsf and the appearance of Tsf on
20 proteinous *E. coli* flocs, our results demonstrated that the design of a fusion protein with
21 Tsf enables displaying a recombinant target protein on the floc structure. Interestingly,
22 the percentage of Tsf-GFP in total floc protein reached approximately 15 % (w/w).

23 To date, many studies have been focusing on the mechanism of microbial flocculation
24 or control of flocculation in terms of external factor such as culture conditions and kind

of flocculants [18]. However, the functionalization of flocs based on the gene modification of microbes has not been reported. In particular, in the case of *E. coli*, the forced flocculation by adding the flocculants was exclusively reported [3, 4]. Therefore, our findings of spontaneous floc formation of *E. coli* and further functionalization by displaying the recombinant protein on the floc structure will give a new concept for the engineering and application of microbial flocs.

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FIGURE LEGENDS

Fig. 1 Floc formation of *E. coli* BW25113/*bcsB*, BW25113/*bcsB/gfp* and BW25113/*bcsB/tsf-gfp* strains. (A) Image of *E. coli* floc suspensions irradiated under fluorescent light (FL) or black light (BL) lamps. The flocs were harvested at 24 h of culture. (B) Floc protein concentration of each *E. coli* strain. (C) Cell number of each *E. coli* strain included in flocs. In graphs (B) and (C), data were obtained from more than three independent experiments. Vertical bars indicate standard deviation.

1 Fig. 2 CLSM images of flocs formed by *E. coli* BW25113/*bcsB*, BW25113/*bcsB/gfp*
2 and BW25113/*bcsB/tsf-gfp* strains. GFP (green) visualized localization in the
3 floc structure, and DNA as a whole bacterial cell's indicator (red) was stained
4 with PI.

1 Fig. 3 Quantification of recombinant proteins expressed on/in flocs and cells of each
2 strain. (A) Western blotting analysis of GFP and Tsf-GFP. Hybridization was
3 conducted using an anti-GFP primary antibody. (B) Contents of recombinant
4 proteins expressed on/in between flocs and cells. Data were obtained from more
5 than three independent experiments. Vertical bars indicate standard deviations.
6 An asterisk indicates the statistical significance as determined by the Student's
7 *t*-test ($p < 0.05$).

Table 1 *E. coli* strains and plasmids used in this study.

Strains or plasmids	Note	Reference
Strains		
BW25113	Host strain of Keio collection	[10]
Plasmids		
pNTR-SD- <i>bcsB</i>	pNTR-SD carrying <i>bcsB</i> under P _{lac} control, Amp ^r	[11]
pCA24N	Cm ^r	[12]
pCA24N- <i>gfp</i>	pCA24N carrying <i>gfp</i> under P _{T5-lac} control, Cm ^r	This study
ASKA- <i>tsf-gfp</i>	pCA24N carrying <i>tsf</i> and <i>gfp</i> under P _{T5-lac} control, Cm ^r	[12]