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Enhanced floc formation by *degP*-deficient *Escherichia coli* cells in the presence of glycerol

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ABSTRACT

Floc formation is an aggregation phenomenon of microbial cells in which the cells form flocs. In this study, it was found that addition of glycerol to a complex glucose medium promoted spontaneous floc formation by an *Escherichia coli degP*-deficient mutant strain (Δ*degP*) in a dose-dependent manner. In the presence of 10% (v/v) glycerol, the amount of floc formation (quantified as floc protein) reached its maximum value (230 mg/L), five times that in its absence. 10% (v/v) glycerol was the limit concentration that does not inhibit cell growth of Δ*degP* strain. Glycerol was not consumed by Δ*degP* cells during floc formation. To provide media having nearly the same viscosity as that containing 10% (v/v) glycerol, carboxymethyl cellulose (CMC) or polyvinylpyrrolidone (PVP) were added to medium as viscosifying agents. Floc formation was not promoted by increasing the medium viscosity with CMC or PVP. However, addition of ethylene glycol also significantly promoted floc formation in the same manner as glycerol. Addition of short-chain polyols decreased the number of viable Δ*degP* cells in the floc structure and enhanced outer membrane vesicle (OMV) production by Δ*degP* cells; polyols-induced damage of outer membrane in Δ*degP* cells may contribute to the promoted floc formation.

INTRODUCTION

Flocculation is an aggregation phenomenon of bacterial cells in which they form flocs or flakes. Numerous microorganisms have been found to have 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 normally lacks floc-forming ability. Many researchers have attempted to induce floc formation by *E. coli* by adding artificial flocculants such as inorganic compounds including aluminum, or cationic polymers such as chitosan (3-5). We previously reported self-produced flocs of *E. coli* cells on overexpression of the native *bcsB* gene which encodes a component of transmembrane cellulose synthase (6), or deletion of *degP*, a periplasmic protease (7, 8). Further experiments into floc formation clarified that the spontaneous *E. coli* flocs were proteinaceous, and there was a strong correlation between spontaneous floc formation and the production of outer membrane vesicles (OMVs) (8). The DegP protein managed unfolded and misfolded proteins (9) and its deletion strongly enhances OMV production in *E. coli* cells (10). Enhanced OMV production in the Δ*degP* cells is a possible strategy that eliminates these undesired proteins from the cell envelope. Compared with forced floc formation, self-generated *E. coli* flocs are promising for application in bioprocessing because cells within the flocs can maintain activity as biocatalysts (11). However, because the amount of floc formation by *E. coli* cells is still low, improvement of floc formation is needed for further application.

In this study, we aimed to increase the amount of floc formed by *E. coli* and understand the mechanism of promoted floc formation. We focused on the viscosity of the medium during floc formation because altered shear stress in media of different viscosities might

affect the amount of floc formation. Initially, glycerol was added to medium as a viscosifying agent. The mechanism of promotion of floc formation by glycerol was further investigated and discussed.

MATERIALS AND METHODS

Bacterial strains and media

E. coli strain K-12 BW25113 and its *degP*-deficient mutant JW0157 (Δ*degP*) were obtained from the National BioResource Project (National Institute of Genetics [NIG], Mishima, Japan) (12). Plasmid pNTR-SD-*bcsB* (13) was provided by the NIG. Strain BW25113 transformed with pNTR-SD-*bcsB*, which spontaneously forms flocs, was named BW25113/*bcsB* (8). To express green fluorescent protein (GFP), the Δ*degP* strain was transformed with pCA24N-*gfp* as described previously (14).

E. coli cells were cultured in lysogeny broth (LB; 10 g/L HiPolypeptone, 5 g/L Bacto™ yeast extract, and 10 g/L NaCl). Culture medium for strains harboring plasmids contained 50 mg/L ampicillin or 50 mg/L chloramphenicol.

Flocculation of each *E. coli* strain

For floc formation, all test cultures were precultured in LB for 18 h at 37°C and then inoculated into test tubes containing 4 mL fresh LB with 6 g/L glucose to an optical density at 600 nm = 0.01, and cultured at 37°C with shaking at 140 strokes/min. When necessary, medium was supplemented with glycerol (0%–14% [v/v]), ethylene glycol (0%–10% [v/v]), carboxymethyl cellulose (CMC; 0–2.5 mg/L), or polyvinylpyrrolidone (PVP; 0–500 mg/L). The recombinant strains were cultured in medium containing isopropyl *β*-D-1-thiogalactopyranoside (1 mM), and ampicillin (50 mg/L) or

chloramphenicol (50 mg/L). After 18 h of culture for floc formation, the test tubes containing the floc and cell suspension were left to stand at room temperature for 15 min. After 15 min, flocs precipitated on the bottom of test tubes and separated from suspension cells in a supernatant. OD_{600} in the supernatant was measured as an indicator to cell growth. The resultant precipitates were resuspended in a 0.9% (w/v) NaCl solution and were left stand for 15 min; this step was repeated two times to remove cells loosely adsorbed onto the flocs. The resultant flocs were transferred into microtubes and photographed using a digital camera after centrifugation (13,400 × *g*, 5 min). For quantitative assays, the resultant flocs were ultrasonicated (20 kHz) to prepare uniformly sized flocs. The amount of protein in the flocs was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., MA, USA) and the value was used as an index of floc amount as described previously (11). The number of viable *E. coli* cells included in the floc structure was estimated by measuring ATP as described elsewhere (11). ATP measurement was conducted using an ATP Luminescent Assay Kit (CheckLite 250 Plus, Kikkoman Corp., Japan) with a Lumat LB 9507 tube luminometer (Berthold Technologies, Germany).

Treatment of flocs with enzymes

The collected flocs were treated with 1.5 mg/mL Pronase (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM Tris buffer (pH 7.5) or 120 U/mL DNase I (Nippon Gene Co., Ltd., Japan) in 40 mM Tris buffer (pH 7.9) at room temperature for a prescribed time.

Medium analysis

The viscosity in culture medium was varied by adding different concentrations of

glycerol, CMC or PVP. The viscosity of the cultures was measured with a vibrational viscometer (SV-10A, A&D, Tokyo, Japan) at 37°C. To measure glucose and glycerol concentrations, 1-mL samples were withdrawn from the culture broth after floc formation. After centrifugation (13,400 × *g*, 5 min), the glucose and glycerol concentrations were determined by high-performance liquid chromatography (HPLC) using an ULTRON PS-80 N column and ultrapure water as the solvent with a flow rate of 1.0 mL/min at 60°C. Glucose and glycerol were detected using a refractive index detector.

Observation of flocs

Flocs were observed using a confocal laser scanning microscope, CLSM (DM6000B) with TCS SP8 software (Leica, Germany). The flocs were formed by the GFP-expressing Δ*degP* strain and the floc sample was fixed in 2.5% glutaraldehyde for 2 h, followed by dehydration with a serial gradient of aqueous ethanol solutions. The samples were observed under excitation at 488 nm.

The specimen was also sputtered with an osmium coat and observed under a scanning electron microscope, SEM (model JSM-6500FS, JEOL, Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization Fourier transform-ion cyclotron resonance mass spectrometry (MALDI FT-ICR MS)

Floc sample (10 μL) was subjected to SDS-PAGE with Coomassie Brilliant Blue (CBB) staining. The protein bands of interest were cut from the gel and subjected to in-gel digestion using trypsin. The digested peptides were analyzed by FT-ICR-MS using MALDI, at the Research Center for Artificial Photosynthesis, Osaka City University

(Osaka, Japan). Spectra were acquired automatically, and then searched against an *E. coli* database from the National Center for Biotechnology Information using the Mascot Server (Matrix Science). Protein scores greater than 77 were judged to be significant ($p<0.05$).

OMV isolation

OMVs were isolated as previously described (15) with some modifications (8). Briefly, after floc formation for 18 h, the flocs and *E. coli* cells were removed from 40 mL of culture broth by centrifugation at 10,000 × *g* for 10 min at 4°C. Then, the supernatant was passed through a 0.45-μm pore filter. Ammonium sulfate was added (final concentration, 400 g/L) for 1 h at room temperature to precipitate the contents. Crude OMVs were obtained by centrifugation at 11,000 × *g* for 30 min at 4°C. The crude extracts were dissolved in 1 mL of 15% (v/v) glycerol solution and then concentrated using a CS100FNX ultracentrifuge (Hitachi Koki Co., Tokyo, Japan) at 109,000 × *g* for 1 h. The OMV pellets were resuspended in 40 μL of 15% (v/v) glycerol solution. The resulting OMV samples were 1000-times more concentrated compared with the original culture broth because of a decrease in the volume from 40 mL to 40 μL. The samples were analyzed by SDS-PAGE with CBB staining. OMV production was quantified as previously described (10) with some modifications. To index the OMV concentration, we photographed the SDS-PAGE bands at ~37 kDa and analyzed the densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA). The index was normalized against OMV production by the wild-type strain. OMVs were also quantified on the basis of phospholipid, with minor modifications from a previously described method (16-18). Briefly, isolated OMVs were incubated with FM4-64 (Molecular

Probes/Thermo Fisher, IL, USA) at a final concentration of 5 μg/mL in phosphate-buffered saline (pH 7.4) for 20 min. We used samples without OMVs or without FM4-64 as negative controls. After excitation at 558 nm, emission at 734 nm was measured using an INFINITE 200 PRO spectrofluorophotometer (TECAN, Switzerland).

RESULTS AND DISCUSSION

Effect of glycerol on *E. coli* floc formation

In this study, we determined the effect of glycerol on the floc formation of *E. coli* Δ*degP* cells and BW25113/*bcsB*. In our previous study, it was shown that the main components of *E. coli* flocs were outer membrane proteins, and determination of the protein concentration could be used as an index of the amount of floc (11). As a result, in the case of Δ*degP* cells, the flocs formed were approximately 1–2 mm in size as reported previously (8). Quantitative evaluation revealed that floc protein without glycerol addition was approximately 50 mg/L, and then showed a dose-dependent increase with glycerol concentration (Fig. 1**A**). Floc protein with 6% (v/v) increased to about 150 mg/L glycerol and reached a maximum value of approximately 230 mg/L at 10% (v/v) glycerol, 5 times higher than that in the absence of added glycerol. Further increase of glycerol (to 12% [v/v]) decreased the amount of floc protein significantly, and 14% (v/v) glycerol completely suppressed floc formation of Δ*degP* cells. To confirm the main component of the Δ*degP* flocs formed with or without glycerol addition (10% [v/v]), flocs were treated with Pronase or DNase I. Following treatment for 5 min, the control (suspended in 0.9% NaCl solution) and DNase I-treated samples did not show any changes (Supplementary Fig. S1). The Δ*degP* flocs were not degraded at all by DNase I even after reacting for 60 min. In contrast, significant degradation of the flocs was observed after 5 min of treatment

with Pronase; degradation became more prominent after 60 min of treatment (Fig. S1). Given that enough DNase I was added to deflocculate DNA-based flocs (19), protein was thought to be main component of the Δ*degP* flocs regardless of glycerol addition.

Considering cell growth, OD_{600} was measured in the supernatant from which flocs were separated by precipitation. As shown in Fig. 1**B**, the OD_{600} value of Δ*degP* cells was ~3 without glycerol addition and addition of glycerol (2%–10% [v/v]) did not significantly change the OD_{600} value. The OD_{600} value slightly decreased at 12% (v/v) and drastically dropped at 14% (v/v). At 14% (v/v) glycerol, the floc formation of Δ*degP* cells was also strongly repressed. These results was thought to be due to the higher osmotic pressure by elevated glycerol concentration (20). Therefore, addition of glycerol at the limit concentration that does not inhibit growth was important for enhancing floc formation of *E. coli* Δ*degP* strain.

In the case of BW25113/*bcsB*, the amount of floc protein was 111 ± 11 mg/L in LB containing 6 g/L glucose. However, for this strain, no floc protein was detected in the presence of glycerol (data not shown). Considering that the growth of BW25113/*bcsB* cells was not inhibited by addition of any concentration of glycerol (data not shown), the effect of glycerol on floc formation of these strains are quite different. The reason glycerol prevented floc formation by BW25113/*bcsB* cells has not been elucidated so far. The effect of glycerol addition on wild-type strain BW25113 was further examined. Strain BW25113 did not form any floc in the presence of glycerol (2%–14% [v/v]) and there was no significant effect of glycerol addition on cell growth (data not shown). These results suggest that the effect of glycerol on floc formation is specific to the Δ*degP* strain. This mutant is more susceptible than wild-type strain to membrane stress because of the

accumulation of misfolded protein in the cell envelope, and there may be some connection between this property and the promotion of floc formation by glycerol.

Glucose and glycerol consumption during Δ*degP* floc formation

In Fig. 1, glycerol was added to check the effect of increased viscosity of medium on the amount of floc formation by *E. coli* Δ*degP* cells. However, we expected that some glycerol would be consumed as a substrate by the cells. Therefore, the amounts of the two main carbon sources in the medium—glucose and glycerol—were measured before and after floc formation (Fig. 2). The initial glucose concentrations were determined to be 5–6 g/L, corresponding to the expected medium composition (6 g/L). The final glucose concentration after culture was about 1.5 g/L in the absence of added glycerol; thus, some glucose remained in the medium after floc formation. Glucose consumption was not different on addition of glycerol (2%–10% [v/v]), suggesting that glucose consumption was not related to the floc formation promoted by glycerol addition. At 12% (v/v) glycerol, the final glucose concentration was about 3 g/L, suggesting that a high concentration of glycerol suppressed glucose consumption by Δ*degP* cells; further addition of glycerol (14% [v/v]) strongly inhibited glucose consumption. Here, decrease of glucose consumption corresponded with decreased floc formation and OD_{600}.

Glycerol concentration in the medium was also determined before and after culture (Fig. 2**B**). The glycerol concentration did not change during the experiment, suggesting that glycerol was not consumed by *E. coli* Δ*degP* cells. Previous research reported that glucose use prevents metabolism of glycerol because of carbon catabolite repression, and the amount of glycerol starts to decrease after glucose is completely consumed (21). Thus, glycerol was probably not consumed during floc formation because some glucose

remained in the cultures. Since glycerol was not consumed at all, the medium viscosity was thought to be high throughout the culture period.

Effect of elevated viscosity on floc formation

It was confirmed that glycerol was not consumed by *E. coli* cells and high viscosity was maintained in the culture medium containing glycerol throughout the culture period. Therefore, we hypothesized that the viscosity of the medium affected the amount of floc formation. To provide a medium having nearly the same viscosity as that containing 10% (v/v) glycerol, CMC or PVP was added to the medium as a viscosifying agent. The relationship between medium viscosity and the amount of floc protein is shown in Fig. 3. The promoting effect of glycerol on floc formation was clearly confirmed with 6% (v/v) glycerol (viscosity 0.9 mPa·s). The maximum value of floc protein was about 230 mg/L, obtained in the presence of 10% (v/v) glycerol (viscosity 1.2 mPa·s). In the case of CMC, the viscosity of the medium increased with increasing CMC addition, and reached about 1.0 mPa·s in the presence of 25 mg/L CMC. However, the amount of floc protein did not significantly increase with CMC addition, and it was 74 mg/L at 1.0 mPa·s. The same trend was observed in the case of PVP; PVP addition also increased the viscosity of the medium, and it reached 1.1 mPa·s at 300 mg/L PVP, but the amount of floc protein was 77 mg/L, similar to that in the control without a viscosifying agent (55 mg/L at 0.7 mPa·s). These results suggest that the promotion of floc formation by glycerol addition is not due to elevation of viscosity in the medium.

Effect of ethylene glycol on *E. coli* floc formation

The results above showed that glycerol in the medium was not consumed by *E. coli* cells, and elevated viscosity was not the reason for the promotion of floc formation by glycerol. Thus, we hypothesized that physicochemical properties of short-chain polyol compounds might promote the floc formation by *E. coli* Δ*degP* cells. Therefore, ethylene glycol was selected as another typical short-chain polyol compound. Figure 4**A** shows the floc formation by Δ*degP* cells in the presence of different concentrations of ethylene glycol. An ethylene glycol-dose-dependent increase of floc formation was observed, similar to that for glycerol. The maximum floc formation (quantified as floc protein) was 210 mg/L in the presence of 8% (v/v) ethylene glycol, comparable to the value seen with 10% (v/v) glycerol. Further addition of ethylene glycol (10% [v/v]) decreased the floc formation drastically. Considering cell growth, OD_{600} of Δ*degP* cells was also measured (Fig. 4**B**). While cell growth was not repressed with ethylene glycol in the range from 2 to 8% (v/v), addition of 10% (v/v) slightly suppressed the cell growth in addition to drastic decrease of floc formation. This is a toxic effect of a high concentration of ethylene glycol, and similar to what we observed in the presence of high concentrations of glycerol. About the action mechanism of short-chain polyols to the floc formation of Δ*degP* cells, since floc formation was not promoted when only glucose concentration was elevated to reproduce nearly the same osmotic pressure (data not shown), the high membrane permeability of short-chain polyols is supposed to be involved in this phenomenon. Moreover, because addition of glycerol did not promote the floc formation of BW25113/*bcsB* cells, it seems that there is a specific action mechanism between polyols and outer membrane of Δ*degP* cells.

From these results, we suggest that the promoted floc formation of Δ*degP* cells is due to the physicochemical properties of short-chain polyols, and addition of polyols at the

limit concentration that does not inhibit growth is important for enhancing floc formation of *E. coli* Δ*degP* strain.

Viable cells in the flocs formed with or without addition of short-chain polyols

To observe the distribution of viable *E. coli* cells within the flocs, we introduced a GFP expression plasmid (pCA24N-*gfp*) (14) into *E. coli* Δ*degP* cells. Figure 5 shows images of typical flocs formed by GFP-expressing Δ*degP* cells with or without added glycerol or ethylene glycol. In the absence of short-chain polyols, optical microscopy showed fibrous structures on the surfaces of flocs (see bright field image in Fig. 5). Three-dimensional images obtained by confocal scanning laser microscopy revealed that GFP-labeled *E. coli* cells were present within the floc structure. Considering that the flocs were washed several times with saline solution before observation, it can be speculated that the viable *E. coli* cells are firmly bound to the flocs.

Meanwhile, in the presence of short-chain polyols (glycerol or ethylene glycol), although the size and the shape of the flocs were very similar to those in the absence of polyol addition, fewer GFP-labeled cells were found in the floc structure. While the total floc amount determined based on the amount of floc protein increased, the number of viable cells within flocs decreased with polyol addition. Subsequently, the number of viable cells per unit weight of floc protein was estimated from the ATP content and compared among conditions. As shown in Fig. 5**B**, the number of viable Δ*degP* cells included in the flocs was about 1.4×10^{9} cells/mg-floc protein in the absence of polyol. In contrast, in the presence of glycerol or ethylene glycol, the number of viable cells was 3.4×10^{7} and 3.6×10^{7} cells/mg-floc protein respectively, supporting the conclusion that addition of polyols decreased the number of viable cells within the flocs. A similar

tendency was also observed by scanning electron microscopy (SEM) (see Supplementary Fig. S2). SEM observation also confirmed the fibrous structures and *E. coli* cells on the flocs. However, zoomed images revealed that the number of cells on the flocs were less with glycerol or ethylene glycol addition, and most of cells were partially or fully damaged compared to those without addition of polyols. Considering that polyols were added at the limit concentration that does not inhibit growth, it is estimated that partially damaged Δ*degP* cells by addition of polyols contributed the construction of floc.

As for cell damage by addition of polyols, there is an important reference that described the plasmolysis of *E. coli* induced by exposure to very high concentrations of glycerol (>50% [v/v]) (20). In that study, the *E. coli* maintained high cell viability (>90%) in the presence of ~50% (v/v) glycerol, and the cytoplasmic membrane was intact. More interestingly, *E. coli* cells produced small endocytotic vesicles in those conditions. Given that the Δ*degP* mutant is more susceptible to membrane stress due to the accumulation of misfolded protein in the cell envelope, it is possible that a similar phenomenon occurred for that strain in a lower glycerol concentration range.

Protein analysis of floc and effect of short-chain polyol addition on Δ*degP* cells

In our previous study, it was determined that *E. coli* flocs induced by *bcsB* overexpression were proteinaceous, and SDS-PAGE followed by nanoLC–MS/MS analyses of the whole flocs revealed that two outer membrane proteins (OmpA, OmpC) and translation elongation factor were the main protein constituents (8). There was also a strong correlation between spontaneous floc formation and the production of OMVs (8). Thus, floc formed by the BW25113/*bcsB* strain was mainly composed of outer membrane of partially damaged *E. coli* cells. Explosive cell lysis is one of the important routes for

the formation of OMVs (22). Thus, it can be speculated that the addition of short-chain polyols induced damage of *E. coli* Δ*degP* cells, which was followed by OMV production.

In the present study, the proteins in the flocs formed by Δ*degP* cells were analyzed by SDS-PAGE (see Supplementary Fig. S3). Regardless of polyol addition, one major band at ~48 kDa and two major bands at ~37 kDa were identified in all samples. MALDI FT-ICR MS analysis identified elongation factor, OmpA and OmpC from these three bands. The presence of these three proteins is consistent with observations of flocs formed by strain BW25113/*bcsB* in previous work (8), suggesting that the components of the flocs in that work and in the present study are very similar, and that OMV production is likely involved in the floc formation. Next, OMVs were isolated from culture broth of Δ*degP* cells with or without polyol addition 18-h post-inoculation using ultracentrifugation. Figure 6**A** shows SDS-PAGE analysis of the OMV sample. Bands at ~37 kDa can be used as indices for quantifying OMV production. We observed that these bands were more intense in cultures with glycerol or ethylene glycol addition compared with culture without added polyol, suggesting that polyol addition promoted OMV production by Δ*degP* cells. Next, the OMV production was analyzed quantitatively based on densitometry of the ~37 kDa band from each condition. As shown in Fig. 6**B**, Δ*degP* cells with glycerol or ethylene glycol addition produced approximately 10 and 6 times more OMVs than cells without polyol addition, respectively. For further confirmation, OMV production was quantified on the basis of the amount of phospholipid using FM4-64 staining. As shown in Fig. 6**B**, relative OMV production in the presence of glycerol or ethylene glycol was 8 and 5 times higher, respectively, than that without polyol addition. Although these values were slightly lower than those obtained by SDS-PAGE, promotion of OMV production was again confirmed. Thus, the addition of short-chain polyols

enhanced OMV production of *E. coli* Δ*degP* cells. From these results, it is proposed that addition of polyols partially damaged outer membrane and enhanced OMV production of Δ*degP* cells, followed by the promotion of floc formation which was mainly composed of outer membrane of *E. coli* cells. In this aspect, further study is needed to elucidate a detailed action mechanism.

In conclusion, addition of glycerol increased floc formation by an *E. coli* Δ*degP* strain in a dose-dependent manner. Glycerol was not consumed by Δ*degP* cells during floc formation, and increase of medium viscosity by glycerol addition was not the reason for the increased floc formation. Addition of ethylene glycol also significantly promoted floc formation. Addition of short-chain polyols decreased the number of viable Δ*degP* cells in the floc structure and enhanced outer membrane vesicle (OMV) production by Δ*degP* cells; polyols-induced damage of outer membrane in Δ*degP* cells may contribute to the promoted floc formation.

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21. **Yao, R., Xiong, D., Hu, H., Wakayama, M., Yu, W., Zhang, X., and Shimizu, K.:** Elucidation of the co-metabolism of glycerol and glucose in *Escherichia coli* by genetic engineering, transcription profiling, and (13)C metabolic flux analysis, Biotechnol. Biofuels, **9**, 175-175 (2016).
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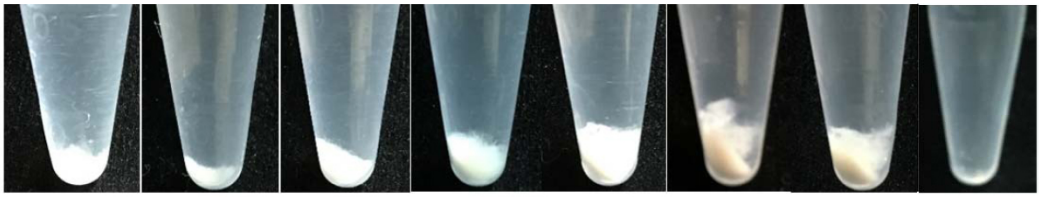
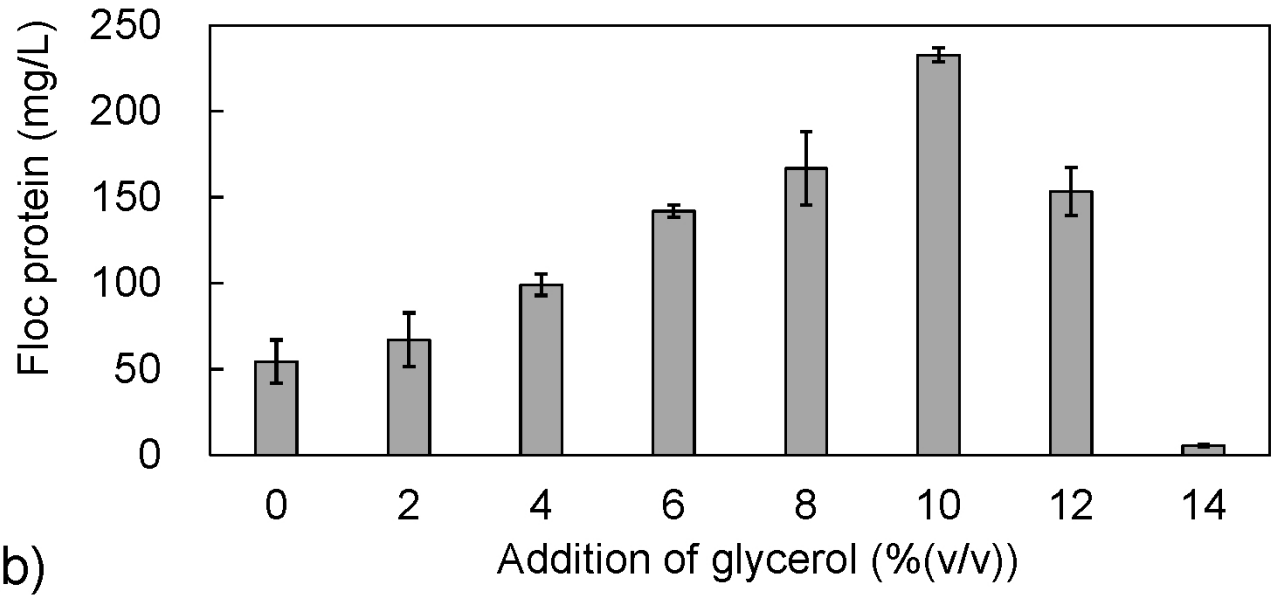
FIGURE LEGENDS

Fig. 1 Floc formation and cell growth of an *Escherichia coli* Δ*degP* strain at different glycerol concentrations. (**A**) Images of *E. coli* flocs in microtubes and floc protein concentration at different glycerol concentrations. The flocs were harvested after 18 h of culture. (**B**) OD_{600} at 18 h. OD_{600} was measured after removing flocs by the precipitation. In (**B**), the data were obtained from >3 independent experiments. Vertical bars indicate standard deviations.

Fig1

(a)




Floc protein (mg/L)
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Addition of glycerol (%(v/v))

(b)

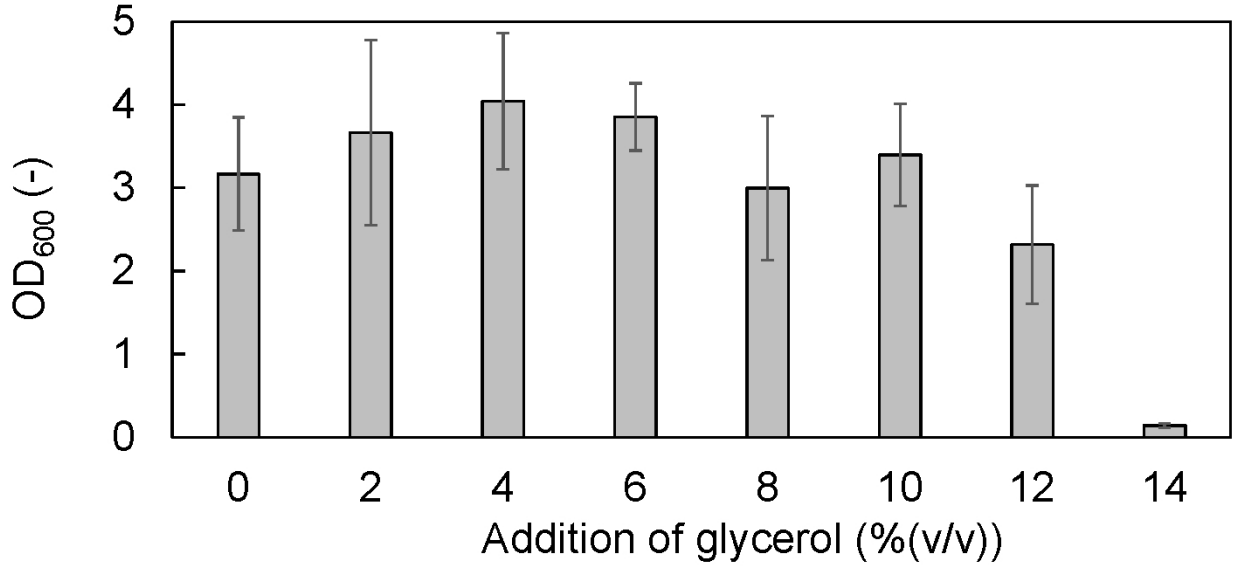
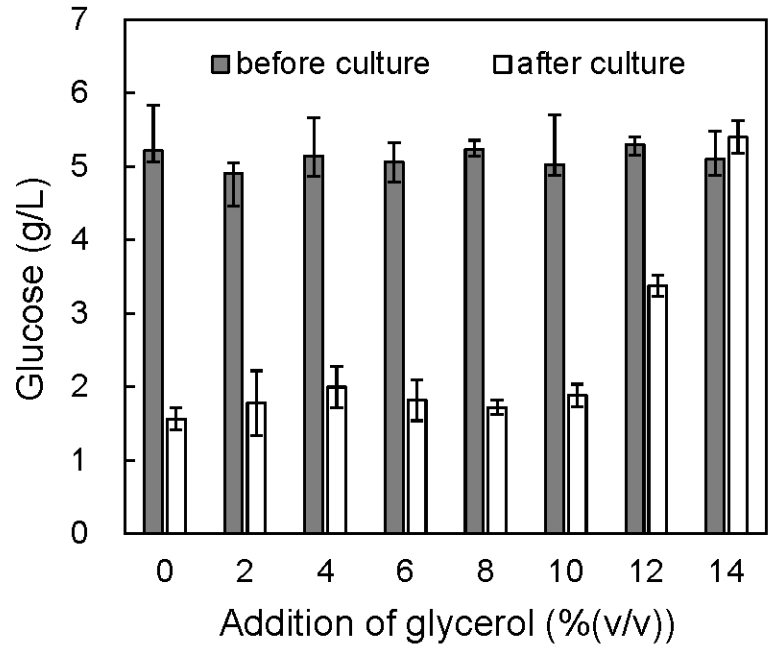

OD600 (-)
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Addition of glycerol (%(v/v))

Fig. 2 Glucose and glycerol concentrations in the medium before and after floc formation of Δ*degP* strain with different concentrations of added glycerol.

Fig2


before culture
after culture
Glucose (g/L)
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
Addition of glycerol (%(v/v))

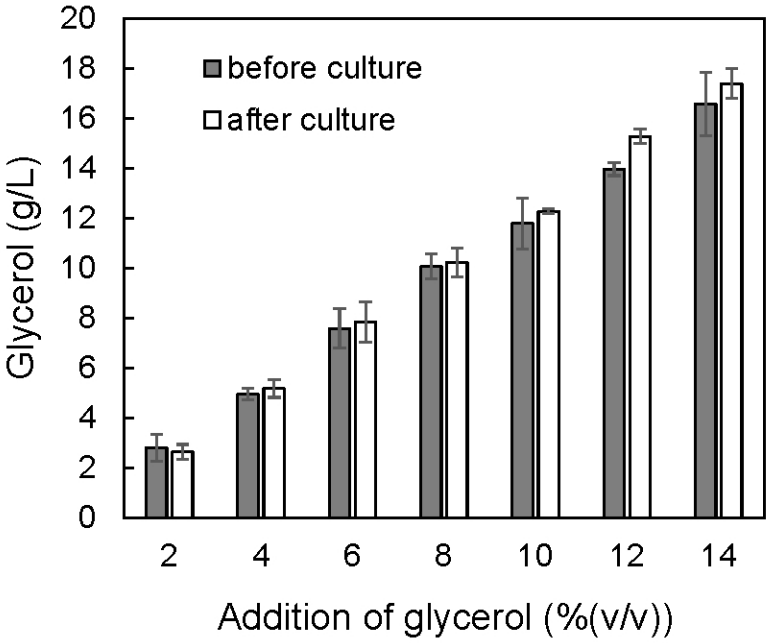

before culture
after culture
Glycerol (g/L)
0
2
4
6
8
10
12
14
16
18
20
2
4
6
8
10
12
14
Addition of glycerol (%(v/v))

Fig. 3 Relationship between medium viscosity and floc protein concentration during floc formation. The viscosity was varied by using different concentrations of glycerol (0%–10% [v/v]), carboxymethyl cellulose (CMC; 0–2.5 mg/L), or polyvinylpyrrolidone (PVP; 0–300 mg/L). Data were obtained from >3 independent experiments and average values are shown.

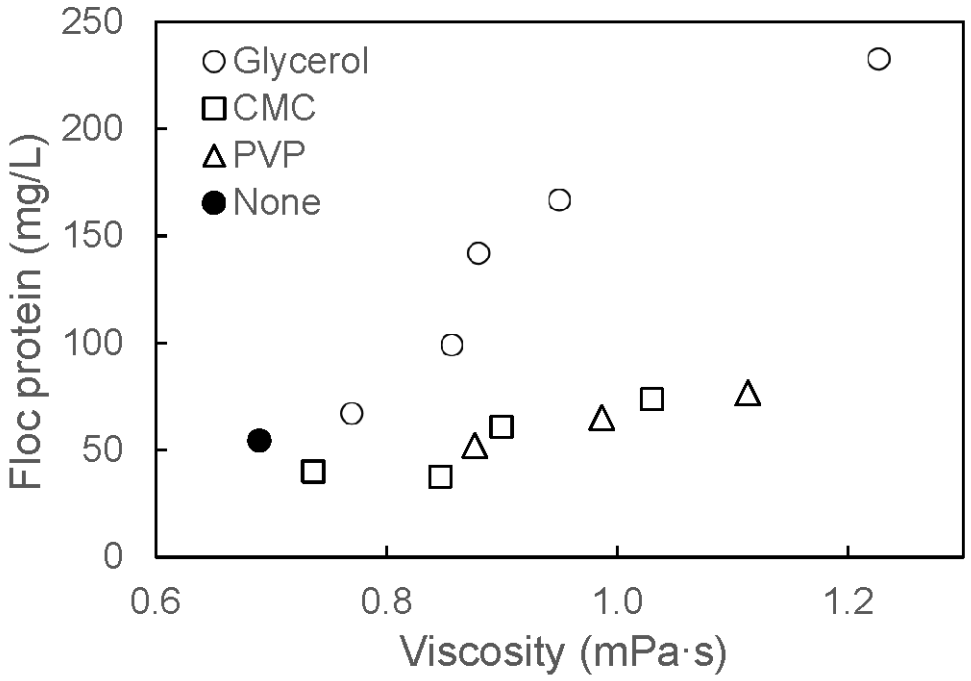
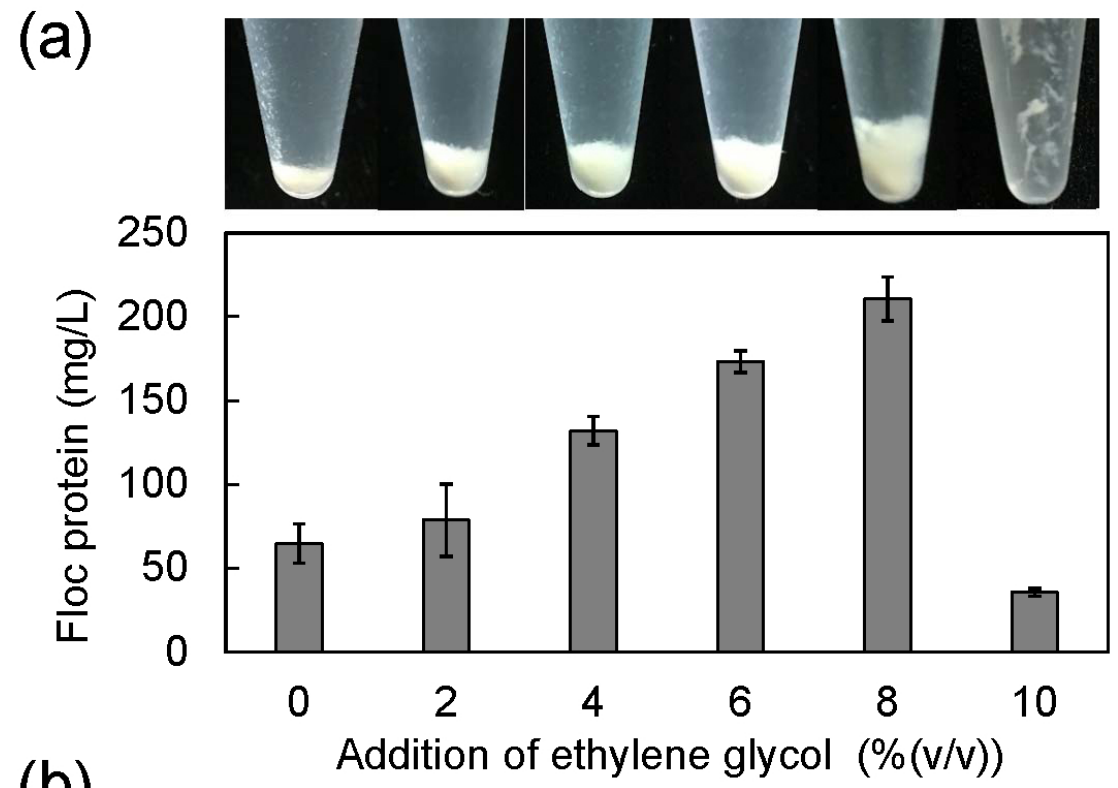


Fig. 4 Floc formation and cell growth of *E. coli* Δ*degP* in the presence of different concentrations of ethylene glycol. (**A**) Images of *E. coli* flocs in microtubes and floc protein concentration at different ethylene glycol concentrations. The flocs were harvested after 18 h of culture. (**B**) OD_{600} at 18 h. In (b), the data were obtained from >3 independent experiments. Vertical bars indicate standard deviations.

Fig4


(a)
Floc protein (mg/L)
250
200
150
100
50
0
0
2
4
6
8
10
Addition of ethylene glycol (%(v/v))

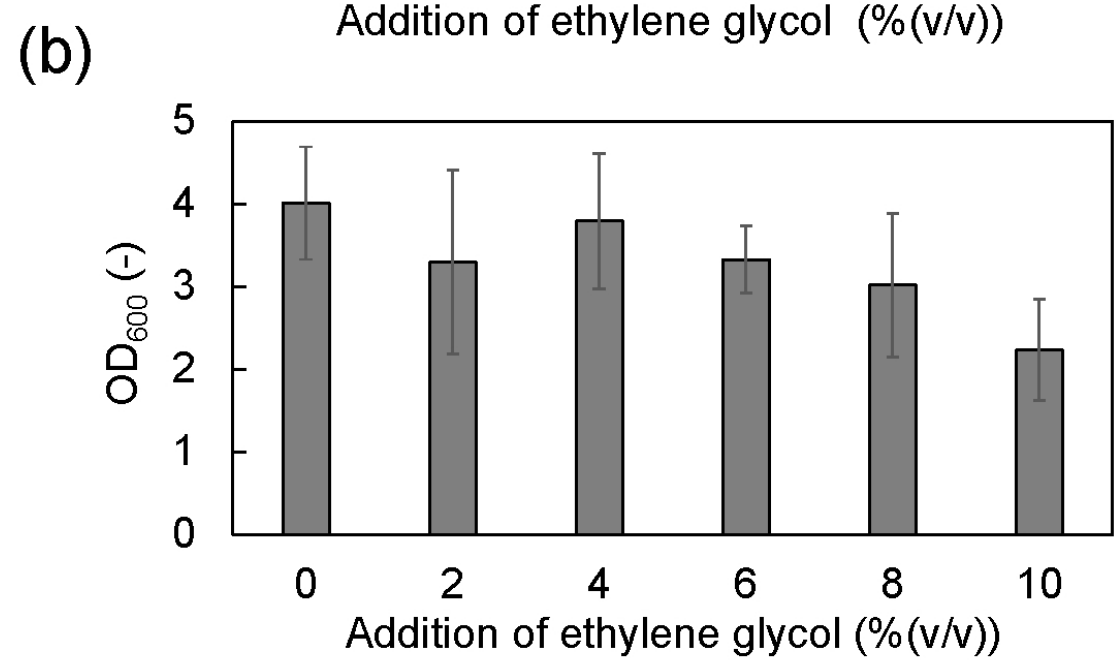

(b)
OD600 (-)
5
4
3
2
1
0
0
2
4
6
8
10
Addition of ethylene glycol (%(v/v))

Fig. 5 Properties of flocs after addition of polyols to culture for 18 h. (**A**) Confocal scanning laser microscopy images of flocs formed with/without glycerol or ethylene glycol. Green fluorescent protein was expressed in the *E. coli* cells to confirm their localization in the floc structure. (**B**) Number of viable Δ*degP* cells included in flocs.

Fig5

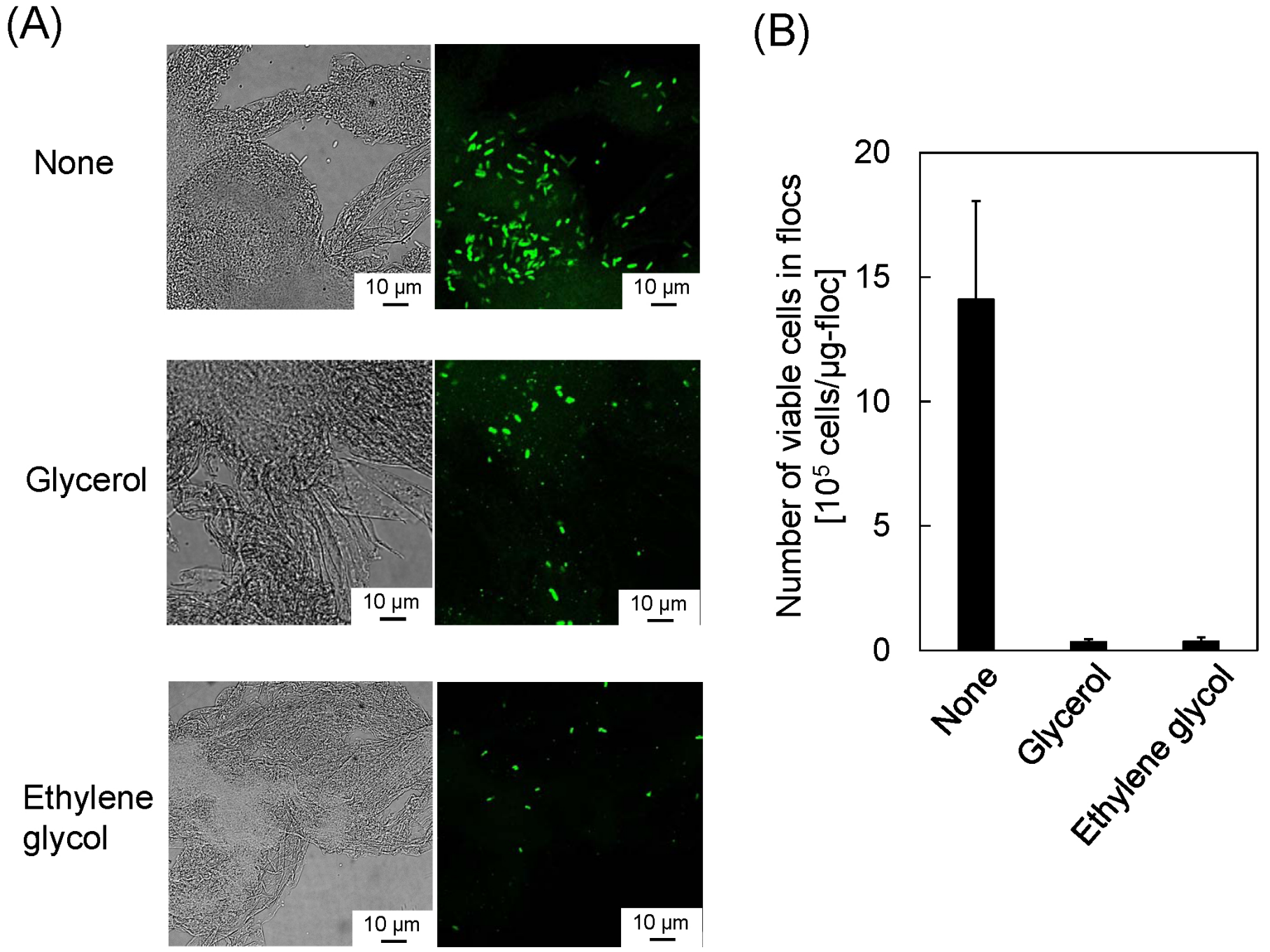
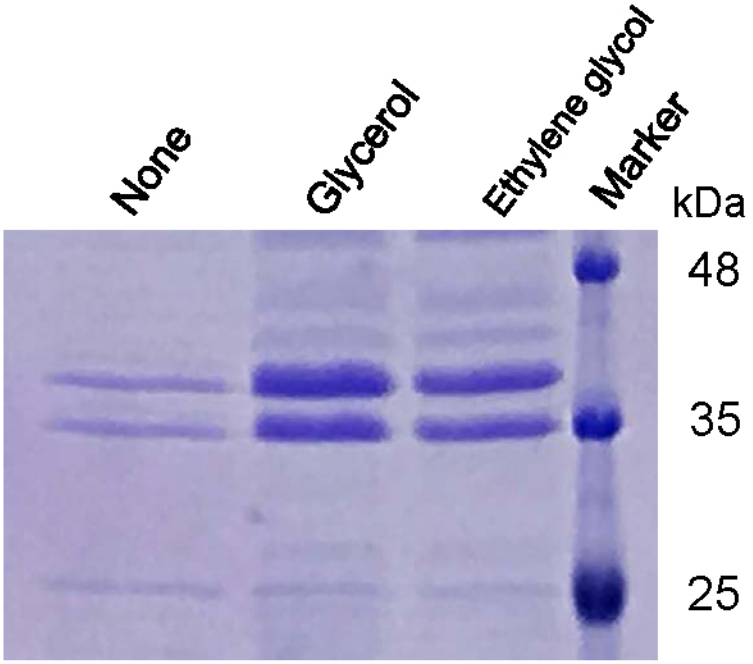

(A)
None
Glycerol
Ethylene glycol
10 μm
10 μm
10 μm
10 μm
10 μm
10 μm
(B)
Number of viable cells in flocs
[10^5 cells/μg-floc]
20
15
10
5
0
None
Glycerol
Ethylene glycol

Fig. 6 Outer membrane vesicle (OMV) production by *E. coli* Δ*degP* cells. (**A**) SDS-PAGE analysis of OMVs isolated from culture broth after floc formation (18 h). (**B**) Relative OMV production in each culture condition quantified based on SDS-PAGE and phospholipid straining with FM4-64. OMV production was normalized to that in the control condition (no added polyol). In (**B**), the data were obtained from >3 independent experiments. Vertical bars indicate standard deviations.

Fig6

(a)


None
Glycerol
Ethylene glycol
Marker
kDa
48
35
25

(b)

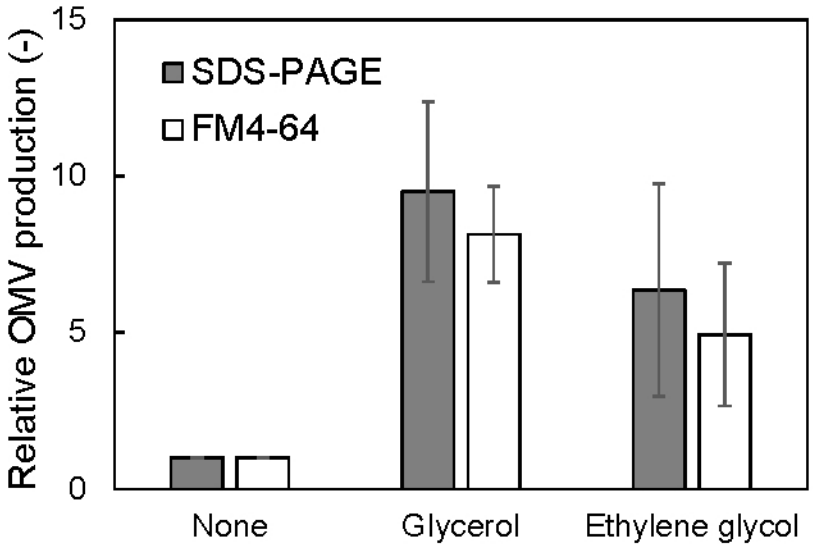

Relative OMV production (-)
15
10
5
0
SDS-PAGE
FM4-64
None
Glycerol
Ethylene glycol

Fig. S1 Treatment of Δ*degP* flocs with Pronase or DNase I. The Δ*degP* flocs were formed with or without glycerol addition (10% [v/v]) before enzyme treatment. In the control test, the flocs were suspended in 0.9% NaCl solution.

Fig. S1

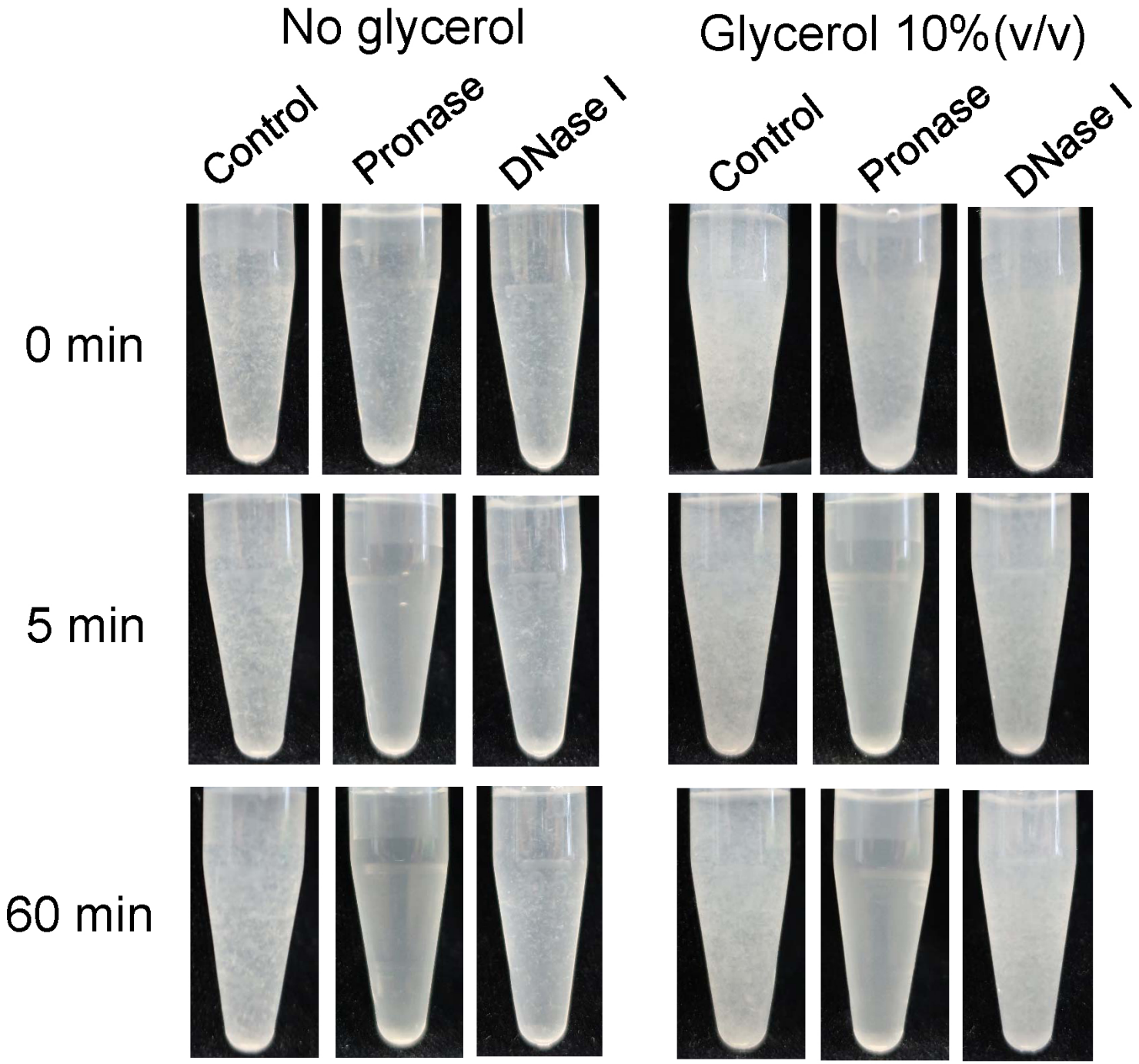

No glycerol
Glycerol 10%(v/v)
Control
Pronase
DNase I
Control
Pronase
DNase I
0 min
5 min
60 min

Fig. S2 Scanning electron microscopy images of Δ*degP* flocs formed with/without glycerol or ethylene glycol.

Fig. S2

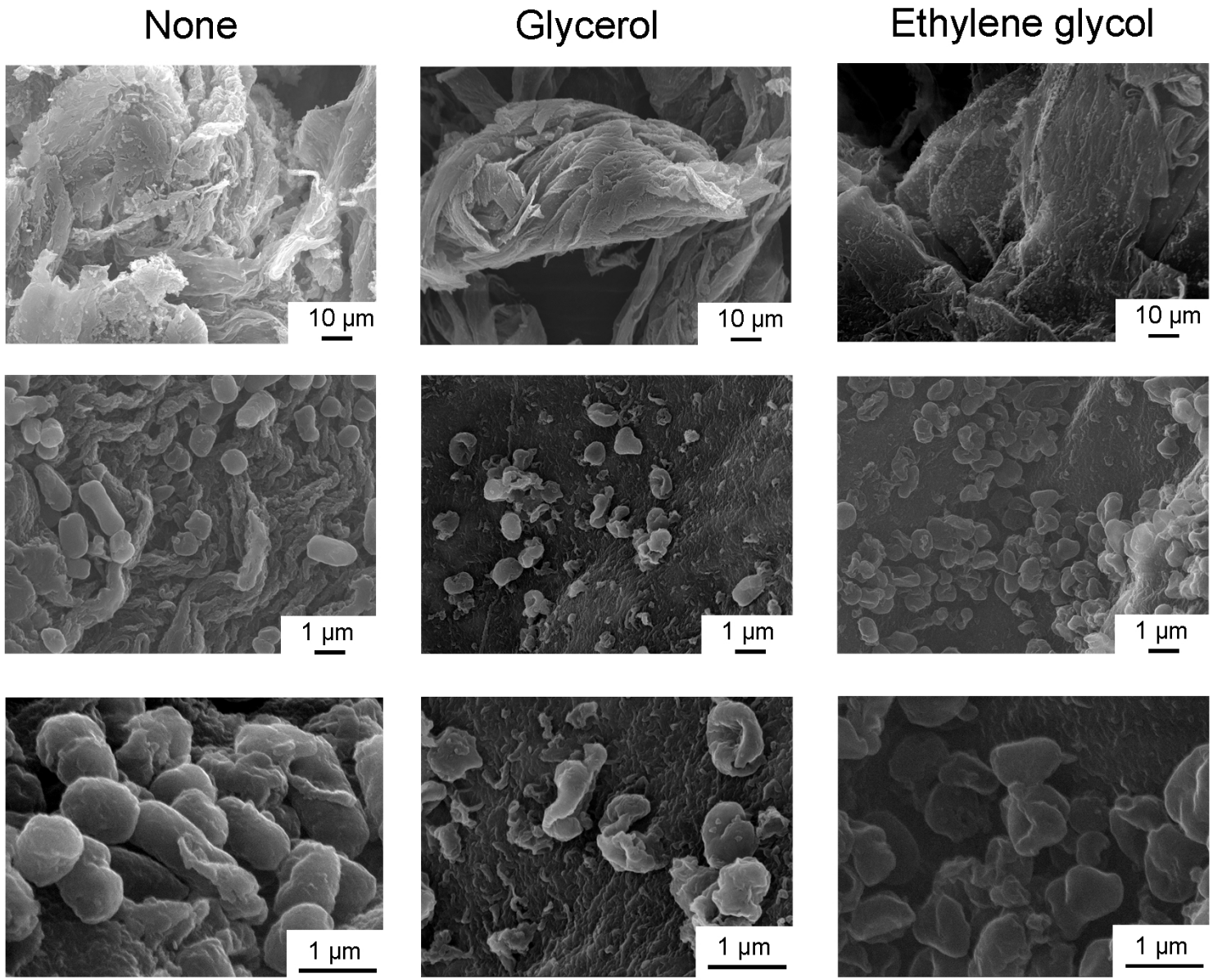
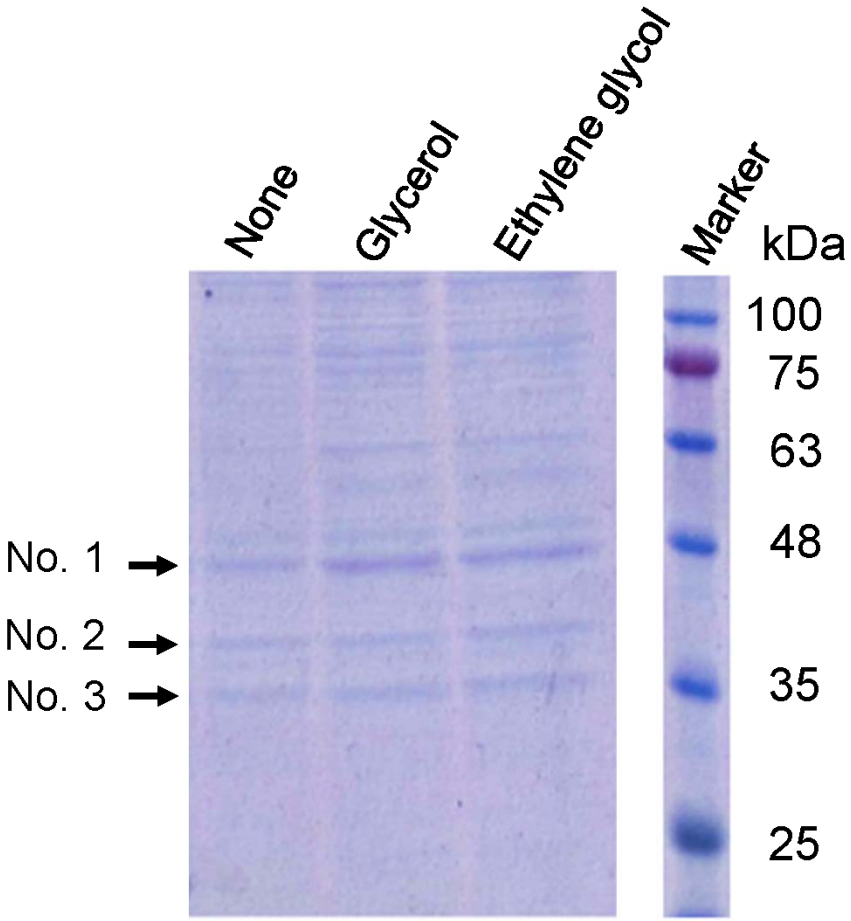

None
Glycerol
Ethylene glycol
10 μm
10 μm
10 μm
1 μm
1 μm
1 μm
1 μm
1 μm
1 μm

Fig. S3 Analysis of floc protein. SDS-PAGE analysis of the flocs with Coomassie Brilliant Blue staining and proteins identified in flocs containing *E. coli* Δ*degP* cells.

Fig. S3



Protein identified in flocs from *E. coli* Δ*degP* cells

No.	Protein	Score
1	Elongation factor Tu	192
2	Outer membrane protein C	175
3	Outer membrane protein A	92