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Creation of a Cellooligosaccharide-Assimilating *Escherichia coli* Strain by Displaying Active Beta-Glucosidase on the Cell Surface via a Novel Anchor Protein▽

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We demonstrated direct assimilation of cellooligosaccharide using *Escherichia coli* displaying beta-glucosidase (BGL). BGL from *Thermobifida fusca* YX (Tfu0937) was displayed on the *E. coli* cell surface using a novel anchor protein named Blc. This strain was grown successfully on 0.2% cellobiose, and the optical density at 600 nm (OD_{600}) was 1.05 after 20 h.

The utilization of biomass as a source of renewable, environmentally friendly energy and/or chemicals has attracted much attention because of the depletion of fossil fuels and increasing environmental problems. Lignocellulosic biomass is regarded as a promising feedstock because it is abundant, inexpensive, and renewable and has favorable environmental properties (11). However, it requires costly and complex hydrolysis steps, such as pretreatment and/or lengthy cellulase treatment (11). Therefore, an efficient and cost-effective method for degradation and fermentation of lignocellulosic biomass into commodity products is required.

Escherichia coli is a promising host for the production of a variety of useful compounds and can metabolize all major sugar monomers existing in plant biomass (1). Metabolic engineering allows for the introduction of desirable pathways to produce target compounds, such as ethanol and other alcohols (2, 4, 10) and lactic acids and other organic acids (3, 17). Since *E. coli* cannot utilize cellobiose and cellooligosaccharides, most compounds are produced from monomeric sugars (i.e., glucose and/or xylose) as carbon sources. Although some *E. coli* isolates can assimilate cellobiose, their slow growth is a major limiting factor (8).

Efficient degradation of cellulose requires a synergistic reaction of the cellulolytic enzymes endoglucanase (EG), cellobiohydrolase (CBH), and beta-glucosidase (BGL). The cellulose is degraded by EG and CBH, resulting in cellobiose and some cellooligosaccharides, which can be converted to glucose by BGL. BGL catalyzes the final step in cellulose degradation as well as stimulates cellulose hydrolysis by relieving the cellobiose-mediated inhibition of EG and CBH (16). Many kinds of EGs have been expressed in *E. coli* for cellulose degradation. However, the enzymatic activity is insufficient for cellulose degradation because of the low secretion of proteins into the medium (15). In the case of BGL, there is only one study in which BGL was expressed using *E. coli* grown on cellobiose (5).

Here, we developed cellooligosaccharide-assimilating *E. coli* by expressing BGL on the cell surface using a novel anchor protein. Generally, the protein-secretive ability of *E. coli* is relatively low compared to those of other microorganisms (15). Alternatively, cell surface display allows for efficient transport of target proteins into the membrane guided by an anchor protein. Therefore, an appropriate anchor protein is an important feature of active protein display (6, 7, 12, 13, 14).

The bacterial strains, oligonucleotides, and plasmids used in this study are listed in Table 1. *E. coli* strain BW25113 (National Institute of Genetics, Japan) and *E. coli* strain JCM20137 (Japan Collection of Microorganisms, RIKEN BRC, which is participating in the National BioResource Project of the MEXT, Japan) were used as host strains. Minimal medium containing 0.2% cellobiose (Sigma-Aldrich Corp., St. Louis, MO) or cellooligosaccharide (Seikagaku Co., Tokyo, Japan) (0.6% Na_2HPO_4, 0.3% K_2HPO_4, 0.05% NaCl, 0.1% NH_4Cl, 1 mM $MgSO_4$, 0.001% thiamine, 0.1 mM $CaCl_2$) was used for growth analysis. The plasmids for BGL expression on the cell surface using the PgsA anchor protein (9) were constructed as follows. The gene encoding BGL from *Clostridium cellulovorans* was amplified by PCR from genomic DNA of *C. cellulovorans* using the BglA_F and BglA_R primers. The amplified gene fragment was digested with BamHI/SpeI and ligated into plasmid pHLA (9). The resultant plasmid was named BglA-pHLA. Other BGL expression vectors were also constructed similarly (Table 1). The gene encoding BGL (Tfu0937) from *Thermobifida fusca* YX were amplified by PCR using the Tfu0937_F and Tfu0937_R primers. The genes encoding BGLs (Sde0245 and Sde2497) from *Saccharophagus degradans* 2-40 were amplified by PCR using the Sde0245_F and Sde0245_R or Sde2497_F and Sde2497_R primer pair. The genes encoding BGLs CHU2268 from *Cytophaga hutchinsonii* and Rumal_1801 from *Ruminococcus albus* were amplified by PCR using the CHU2268_F and CHU2268_R or Rumal_1801_F and Rumal_1801_R primer pair. Each of the amplified fragments was digested by XhoI/HindIII (Tfu0937), BamHI/XhoI (Sde0245, CHU2268, and Rumal_1801), or BamHI/SpeI (Sde2497) and ligated into plasmid pHLA. The

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TABLE 1. Strains, DNA, plasmids, and primers used in this study

Strain, genomic DNA, plasmid, or primer	Relevant phenotype, description, or sequence (5′–3′)[a]	Source or reference
Strains		
Escherichia coli		
NovaBlue	*endA1 hsdR17*(r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proAB*$^+$ *lacI*^{q}ZΔM15::Tn*10* (Tet^r)]; host for DNA manipulation	Novagen
BW25113	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	National Institute of Genetics, Japan
JCM20137	B strain	Japan Collection of Microorganisms, RIKEN BRC
Genomic DNA		
Clostridium cellulovorans	Genomic DNA was prepared by standard DNA extraction method from *Clostridium cellulovorans* 743B (ATCC 35296)	ATCC
Thermobifida fusca	YX (ATCC BAA-629D-5)	ATCC
Saccharophagus degradans	2-40 (ATCC 43961D-5)	ATCC
Cytophaga hutchinsonii	ATCC 33406D-5	ATCC
Ruminococcus albus	ATCC 27210	ATCC
Plasmids		
pHLA	Vector under HCE promoter control; carrying PgsA anchor protein	10
BglA-pHLA	Vector for BGL (BglA) expression using PgsA anchor protein	This study
Tfu0937-pHLA	Vector for BGL (Tfu0937) expression using PgsA anchor protein	This study
Sde0245-pHLA	Vector for BGL (Sde0245) expression using PgsA anchor protein	This study
Sde2497-pHLA	Vector for BGL (Sde2497) expression using PgsA anchor protein	This study
CHU2268-pHLA	Vector for BGL (CHU2268) expression using PgsA anchor protein	This study
Rumal_1801-pHLA	Vector for BGL (Rumal_1801) expression using PgsA anchor protein	This study
Blc-Tfu0937-pHLA	Vector for Tfu0937 expression using Blc anchor protein; the C terminus of Blc was fused to the N terminus of Tfu0937	This study
Slp-Tfu0937-pHLA	Vector for Tfu0937 expression using Slp anchor protein; the C terminus of Blc was fused to the N terminus of Tfu0937	This study
HdeD-Tfu0937-pHLA	Vector for Tfu0937 expression using HdeD anchor protein; the C terminus of Blc was fused to the N terminus of Tfu0937	This study
Tfu0937-Blc-pHLA	Vector for Tfu0937 expression using Blc anchor protein; the N terminus of Blc was fused to the C terminus of Tfu0937	This study
Tfu0937-Slp-pHLA	Vector for Tfu0937 expression using Slp anchor protein; the N terminus of Blc was fused to the C terminus of Tfu0937	This study
Tfu0937-HdeD-pHLA	Vector for Tfu0937 expression using HdeD anchor protein; the N terminus of Blc was fused to the C terminus of Tfu0937	This study
Oligonucleotide primers		
BglA_F	CGCGGATCCATGGAAAAGCTAAGATTTCCCAAAGATTTTATTTTT	
BglA_R	GGACTAGTTTATAAATCTTCTTCACTAATTAATTTTTGTTCCTTATTA GATCTTTCTATAAGCTCCTTATACCAATAAG	
Tfu0937_F	CGACTCTAGACTCGAGGTGACCTCGCAATCGACGACTCCTCTGGGC AATCTGGAGGAGACTCCCAAACCGGATATCCGC	
Tfu0937_R	CAAAACAGCCAAGCTTCTATTCCTGTCCGAAGATTCCCCCGTTGCGC ATCACCCGGGAGTACCACCAGCCGCTGTCC	
Sde0245_F	GCGGATCCATGCTCAAAAAGATAAACAAGAAAGGTCTTGCTTT AAGC	
Sde0245_R	CGAGCTCGAGTTAGTCACACTTAATAGCTGCGCTATCTGCACCGC CAGG	
Sde2497_F	GCGGATCCATGAAAAATACTTTATCCTTTAAAACATCCTTGCTTGC GGGC	
Sde2497_R	GCATACTAGTGTCGACCTATTCGCCCAGCATTTTTTTAAGGGTGGGT TCCATCGC	
CHU2268_F	GCGGATCCATGAAAAAAATAACCGTATTGATTTCCATCTGGCTCAGT GCAGCCGC	
CHU2268_R	ATAGCTCGAGTTACTCATTAAAATATATTTCTGTCTGGAGATTTCCG	
Rumal_1801_F	GCTGGATCCATGATAAAGCTTGATTGGAACGAATATCTCGAAAAGG CAGCAGAGGTAAACGC	
Rumal_1801_R	ATGCCTCGAGTTAATCGATAAGCACGGCGTCCTCGAAGCAGCTTTTT ACTTTGAAAGTTCGC	
Tfu0937_F2	AAAAGACCAGATCTGGCGGCCGCTACCTCGCAATCGACGACTCC TCTG	
Tfu0937_R2	ACAGCCAAGCTTCTAAGCCTTATCGTCGTCATCCTTGTAATCGGATC CTTCCTGTCCGAAGATTCCCCCGTTGCG	

Continued on following page

TABLE 1—*Continued*

Strain, genomic DNA, plasmid, or primer	Relevant phenotype, description, or sequence (5′–3′)	Source or reference
Tfu0937_F3	TGGAAAAAGGAGATCTGATGGTGACCTCGCAATCGACGACTCCTCT GGGCAATCTGGAGGAGACTCCCAAACCGGATATC	
Tfu0937_R3	CAAAACAGCCAAGCTTTTAAGCGGCCGCCTCGAGTTCCTGTCCGAA GATTCCCCCGTTGCGCATCACCCGGGAGTACC	
Blc_F1	TGGAAAAAGGAGATCTGATGCGCCTGCTCCCTCTCGTTGCCGCAGC GACAGCTGCATTTCTGGTCGTTGCC	
Blc_R1	TTGCGAGGTAGCGGCCGCACTACCAGGCTGCTGTACCCAAATAAAT TTACTGACATCAAACCCTTCCCGGGTCGCG	
HdeD_F1	TGGAAAAAGGAGATCTGATGTTATATATAGATAAGGCAACAATTTT GAAGTTTGATCTGGAGATGC	
HdeD_R1	TTGCGAGGTAGCGGCCGCTTGCTGCTTAACGAACAAACTGGCGAAG CTGAACAGGCTGGCGGCGC	
Slp_F1	TGGAAAAAGGAGATCTGATGAACATGACAAAAGGTGCACTCATCCT CAGCCTTTCATTTTTGC	
Slp_R1	TTGCGAGGTAGCGGCCGCTTTGACCAGCTCAGGTGTTACCTGACTC ACCGCATTGGTGTAGTAAGGCGC	
Blc_F2	CGGACAGGAACTCGAGATGCGCCTGCTCCCTCTCGTTGCCGCAGCG ACAGCTGCATTTCTGGTCGTTGCC	
Blc_R2	AGCTTTTAAGCGGCCGCCTAACTACCAGGCTGCTGTACCCAAATAAA TTTACTGACATCAAACCCTTCCCGGG	
Slp_F2	CGGACAGGAACTCGAGATGAACATGACAAAAGGTGCACTCATCCTC AGCCTTTCATTTTTGCTTGCCGC	
Slp_R2	AGCTTTTAAGCGGCCGCTTATTTGACCAGCTCAGGTGTTACCTGACT CACCGCATTGGTGTAGTAAGGCGC	

[a] HCE, high-level constitutive expression.

resultant plasmids were named Tfu0937-pHLA, Sde0245-pHLA, Sde2497-pHLA, CHU2268-pHLA, and Rumal_1801-pHLA (Table 1). All plasmids were introduced into *E. coli* BW25113 or JCM20137 by electroporation using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's procedure.

These BGL activities on the cell surface or in culture medium were quantitatively evaluated using *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Nacalai Tesque, Inc., Kyoto, Japan) as a substrate. One unit of beta-glucosidase activity was defined as the amount of enzyme producing 1 μmol/min *p*-nitrophenol at 37°C and pH 5.0. As shown in Fig. 1A, PgsA-BglA showed the highest BGL activity (294 U/optical density at 600 nm [OD_{600}]/ml) in the case of *E. coli* BW25113, followed by PgsA-Rumal_1801 (103 U/OD_{600}/ml) and PgsA-Tfu0937 (18 U/OD_{600}/ml). In the case of *E. coli* JCM20137 (Fig. 1B), PgsA-BglA also showed the highest BGL activity (293 U/OD_{600}/ml). In all strains, BGL activity on the cell surface was higher than that in the culture supernatant (under 0.0001 U/ml), showing that BGL was successfully expressed on the cell surface and retained its enzymatic function through the PgsA anchor protein.

Using these strains, cell growth on 0.2% cellobiose as the sole carbon source was evaluated (data not shown). Interestingly, *E. coli* displaying Tfu0937 showed the highest growth compared to the other strains, even though the BGL activity of Tfu0937 was not the highest among them (Fig. 1A and B). The final OD_{600} values after 48 h of cultivation were approximately 0.41 for *E. coli* BW25113 and 0.85 for *E. coli* JCM20137. In the case of strains displaying BglA or Sde2497, the OD_{600} values after 48 h of cultivation were 0.24 (BglA) and 0.31 (Sde2497) for *E. coli* BW25113 and 0.38 (BglA) and 0.41 (Sde2497) for *E. coli* JCM20137. The strain carrying the pHLA control plasmid only did not have an increased OD_{600} (0.18 for *E. coli* BW25113 and 0.16 for *E. coli* JCM20137). These results clearly demonstrate that Tfu0937 was an appropriate BGL for cellobiose assimilation in *E. coli*.

We developed a novel anchor protein appropriate for highly active BGL display to improve the cellobiose-assimilating ability. Unexpectedly, previously reported anchor proteins, such as LamB, OmpT, and OmpL (6, 13), did not work for active BGL display (data not shown). Hence, novel anchor proteins Blc, Slp, and HdeD derived from *E. coli* were selected. The plasmids for Tfu0937 expression on the cell surface using various anchor proteins were constructed as follows. The gene fragment encoding Tfu0937 was amplified by PCR from *T. fusca* YX genomic DNA using the Tfu0937_F2 and Tfu0937_R2 primers. The amplified fragment was digested with BglII/HindIII and ligated into plasmid pHLA. The resultant plasmid was named ΔPgsA-c-Tfu0937-pHLA. The genes encoding Blc, Slp, and HdeD were amplified using the following primer pairs Blc_F1 and Blc_R1, Slp_F1 and Slp_R1, and HdeD_F1 and HdeD_R1, respectively. Each amplified fragment was digested with BglII/NotI and ligated into plasmid ΔPgsA-c-Tfu0937-pHLA. The resultant plasmids were named Blc-Tfu0937-pHLA, Slp-Tfu0937-pHLA, and HdeD-Tfu0937-pHLA. In these constructions, the C terminus of the anchor protein was fused to the N terminus of Tfu0937 (Table 1).

As shown in Fig. 1C and D, Blc-Tfu0937 showed the highest BGL activity, 1,140 U/OD_{600}/ml for *E. coli* BW25113 and 7,446 U/OD_{600}/ml for *E. coli* JCM20137. The BGL activity of Blc-Tfu0937 was approximately 65-fold higher than that of PgsA-Tfu0937 in both *E. coli* strains. These results suggest that the specific activity of the Blc-Tfu0937 fusion protein might be improved rather than increasing the amount of protein expressed, because the amounts of fusion proteins (i.e., PgsA-

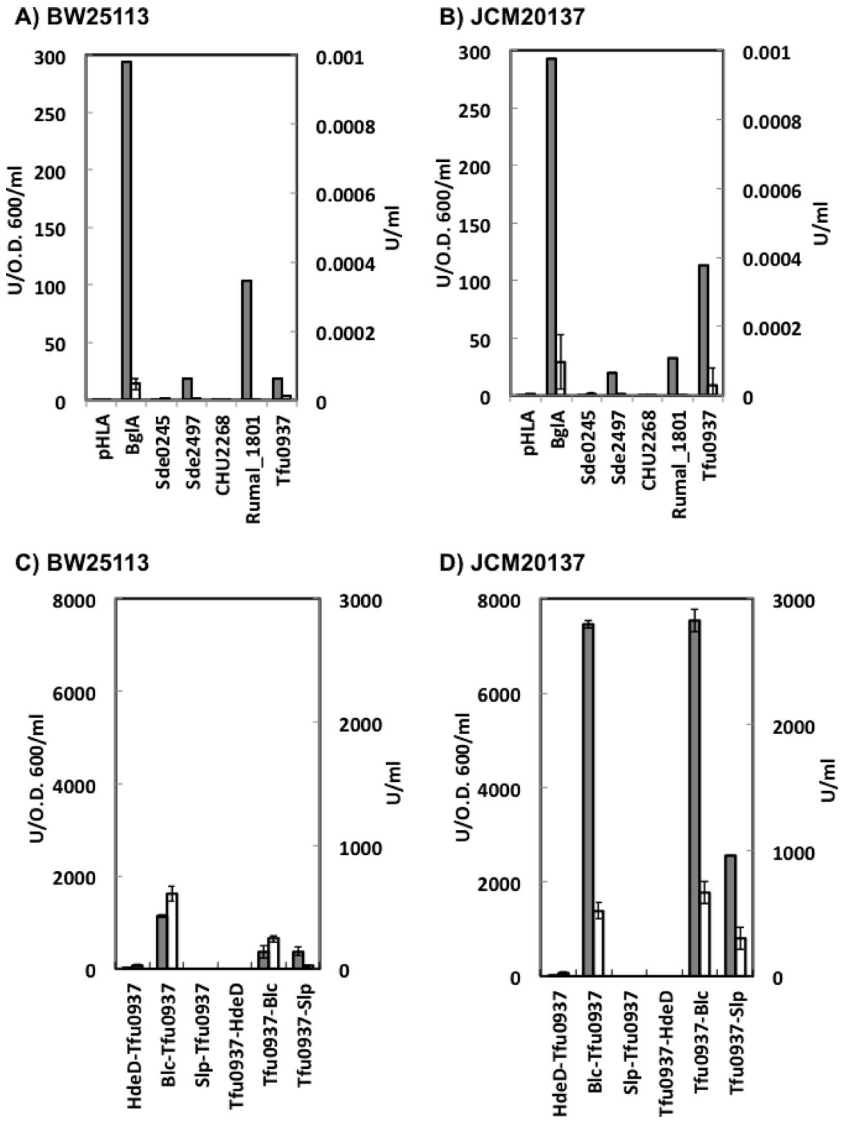

FIG. 1. BGL activity on the *E. coli* cell surface or in culture supernatant after 24 h of cultivation in LB medium. Gray bars show BGL activity on the cell surface (U/OD_{600}/ml; left axis), and white bars show BGL activity in the culture supernatant (U/ml; right axis). (A and B) Each BGL was displayed on the *E. coli* cell surface using the PgsA anchor protein. (A) *E. coli* BW25113; (B) *E. coli* JCM20137. (C and D) Tfu0937 was displayed using various anchor proteins. In the case of HdeD-Tfu0937, Blc-Tfu0937, and Slp-Tfu0937, the C terminus of each anchor protein was fused to the N terminus of Tfu0937. In the case of Tfu0937-HdeD, Tfu0937-Blc, and Tfu0937-Slp, the N terminus of each anchor protein was fused to the C terminus of Tfu0937. (C) *E. coli* BW25113; (D) *E. coli* JCM20137. All data are averages from three independent experiments, and error bars represent standard deviations.

Tfu0937 and Blc-Tfu0937) evaluated by Western blotting were at almost the same levels (data not shown). As expected, the BGL activity on the cell surface was higher than that in the culture supernatant (605 U/ml in *E. coli* BW25113 and 520 U/ml in *E. coli* JCM20137), showing that BGL was expressed successfully on the cell surface and retained its enzymatic function through the novel anchor protein.

The site of the BGL/anchor protein fusion (i.e., N or C terminus) is an important determinant of the displayed BGL activity. We prepared Tfu0937-Blc, Tfu0937-Slp, and Tfu0937-HdeD, in which the C terminus of Tfu0937 was fused to the N terminus of the anchor protein Blc, Slp, or HdeD. The gene fragment encoding Tfu0937 was amplified by PCR from *T. fusca* YX genomic DNA using the Tfu0937_F3 and Tfu0937_R3 primers. The amplified fragment was digested with BglII/HindIII and ligated into plasmid pHLA. The resultant plasmid was named ΔPgsA-n-Tfu0937-pHLA. The genes encoding Blc, Slp, and HdeD were amplified using the following primer pairs Blc_F2 and BlcR2, Slp_F2 and Slp_R2, and HdeD_F2 and HdeD_R2, respectively. Each amplified fragment was digested with XhoI/HindIII and ligated into plasmid ΔPgsA-n-Tfu0937-pHLA. The resultant plasmids were named Tfu0937-Blc-pHLA, Tfu0937-Slp-pHLA, and Tfu0937-HdeD-pHLA. In these constructions, the N terminus of the anchor protein was fused to the C terminus of Tfu0937. Their pNPG degradation activities were evaluated (Fig. 1C and D). In the

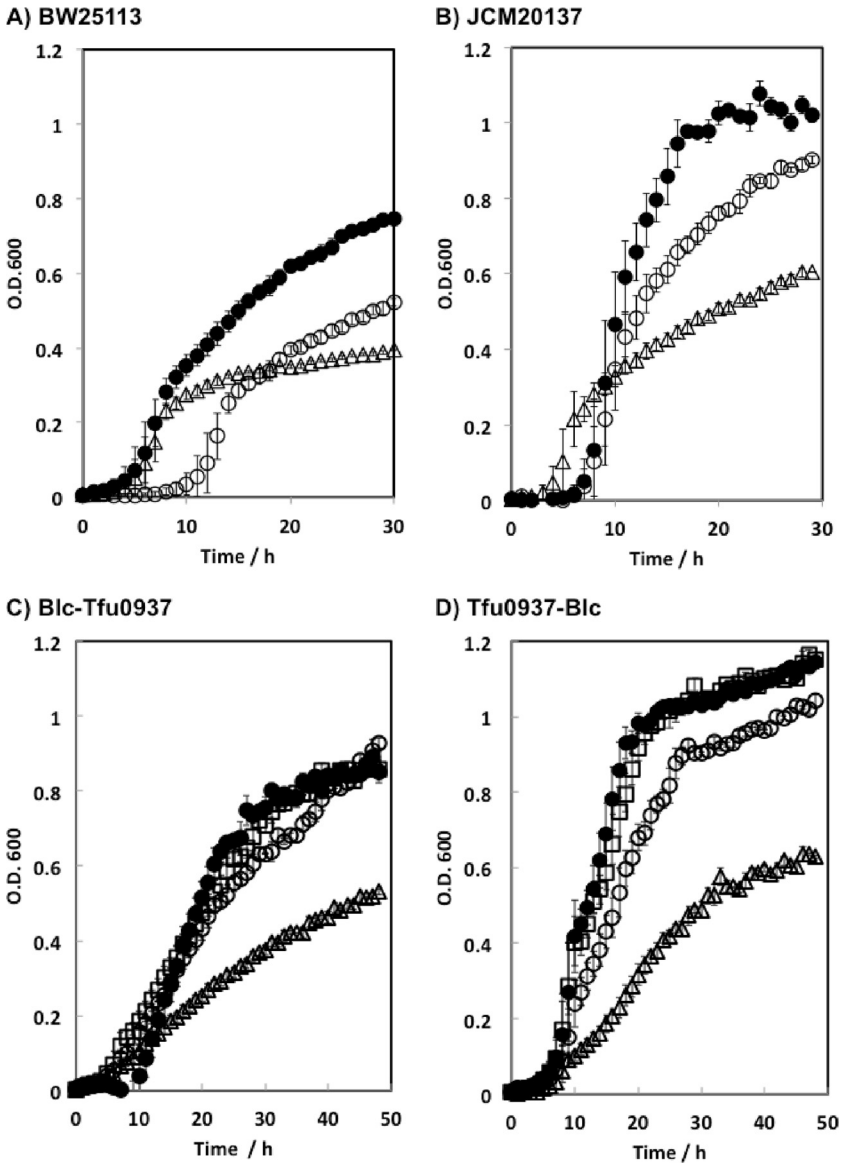

FIG. 2. Growth analysis of Tfu0937-displaying *E. coli* using cellobiose (A and B) or cellooligosaccharide (C and D) as the sole carbon source. (A) *E. coli* BW25113; (B) *E. coli* JCM20137. Symbols shown in panels A and B are as follows: Blc-Tfu0937 (closed circles), Tfu0937-Blc (open circles), and Tfu0937-Slp (triangles). Symbols shown in panels C and D are as follows: cellotriose (closed circles), cellotetraose (squares), cellopentaose (open circles), and cellohexaose (triangles). Data are averages from three independent experiments, and standard deviations were within 10%.

case of JCM20137 (Fig. 1D), Tfu0937-Blc showed high activity (7,535 U/OD_{600}/ml), the same as that of Blc-Tfu0937. These results show that Blc enables the display of target proteins regardless of whether a fusion protein is achieved at the N or the C terminus. In the case of *E. coli* BW25113 (Fig. 1C), low activity was found for both Tfu0937-Blc (353 U/OD_{600}/ml) and Tfu0937-Slp (377 U/OD_{600}/ml). HdeD showed no BGL activity when it was fused to the C terminus of Tfu0937 as an anchor protein.

The growth on 0.2% cellobiose as the carbon source was evaluated. The initial OD_{600} was adjusted to 0.01. The time course analysis of OD_{600} during cultivation at 37°C is shown in Fig. 2A and B. In the case of *E. coli* BW25113 (Fig. 2A), Blc-Tfu0937 showed high growth on cellobiose, followed by Tfu0937-Blc and Tfu0937-Slp. The values of OD_{600} after 30 h of cultivation were 0.75 (Blc-Tfu0937), 0.50 (Tfu0937-Blc), and 0.38 (Tfu0937-Slp). In the case of *E. coli* JCM20137 (Fig. 2B), the OD_{600} of Blc-Tfu0937 was up to 1.05 after 20 h of cultivation, which was a significantly improved growth rate and the same as its growth on 0.2% glucose after 16 h of cultivation (data not shown). The OD_{600} of Tfu0937-Blc also reached 0.9 after 30 h of cultivation, followed by Tfu0937-Slp. Two types of *E. coli* strains were used in this study to show the versatility of BGL display. The cell growth of *E. coli* BW25113 was lower than that of *E. coli* JCM20137, which might be due to the specific character of each *E. coli* strain, because the growth of

JCM20137 on glucose was slightly superior to that of BW25113 (data not shown).

Encouraged by these findings, cell growth on 0.2% cellooligosaccharide (cellotriose, cellotetraose, cellopentaose, or cellohexaose) as the sole carbon source was evaluated (Fig. 2C and D). Tfu0937-Blc showed high growth on each cellooligosaccharide, followed by Blc-Tfu0937. In the case of Tfu0937-Blc, the values of OD_{600} after 30 h of cultivation were 1.03 (cellotriose), 1.04 (cellotetraose), 0.90 (cellopentaose), and 0.49 (cellohexaose). These results clearly demonstrate that Tfu0937-Blc is appropriate for cellooligosaccharide assimilation in *E. coli* JCM20137.

In conclusion, we developed BGL-displaying *E. coli* using the novel anchor protein Blc and successfully demonstrated direct growth on cellobiose and cellooligosaccharide. Optimization of BGL, the anchor protein, and the *E. coli* strain can massively improve both BGL activity and cellobiose-assimilating ability. To date, engineered *E. coli* has produced many kinds of useful compounds from glucose or xylose as a carbon source (2, 3, 4, 10, 17). Our results suggest the possibility to produce useful compounds from cellooligosaccharide as well as glucose. In previous reports, we also have developed a *Saccharomyces cerevisiae* (yeast) cell surface display system and have demonstrated ethanol production from cellulose directly (7, 12). The codisplay of more than one enzyme, such as BGL, EG, and CBH, is required for direct assimilation of cellulose. Our approach will be also useful for other kinds of cellulases, such as EG and/or CBH, and we are currently undergoing further study along these lines.

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