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Bacterial Cell Surface Display of a Multifunctional Cellulolytic Enzyme Screened from a Bovine Rumen Metagenomic Resource

Kyong-Cheol Ko†, Binna Lee†, Dae-Eun Cheong, Yunjon Han, Jong Hyun Choi*, and Jae Jun Song*

Industrial Microbiology and Bioprocess Research Center, Integrated Biorefinery Research Institute, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 580-185, Republic of Korea

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**Corresponding authors*
J.H.C.
Phone: +82-63-570-5150;
Fax: +82-63-570-5109;
E-mail: jhchoi@kribb.re.kr
J.J.S.
Phone: +82-63-570-5140;
Fax: +82-63-570-5109;
E-mail: jjsong@kribb.re.kr

†These authors contributed equally to this work.

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A cell surface display system for heterologous expression of the multifunctional cellulase, CelEx-BR12, in *Escherichia coli* was developed using truncated *E. coli* outer membrane protein C (OmpC) as an anchor motif. Cell surface expression of CelEx-BR12 cellulase in *E. coli* harboring OmpC-fused CelEx-BR12, designated MC4100 (pTOCBR12), was confirmed by fluorescence-activated cell sorting and analysis of outer membrane fractions by western blotting, which verified the expected molecular mass of OmpC-fused CelEx-BR12 (~72 kDa). Functional evidence for exocellulase activity was provided by enzymatic assays of whole cells and outer membrane protein fractions from *E. coli* MC4100 (pTOCBR12). The stability of *E. coli* MC4100 (pTOCBR12) cellulase activity was tested by carrying out repeated reaction cycles, which demonstrated the reusability of recombinant cells. Finally, we showed that recombinant *E. coli* cells displaying the CelEx-BR12 enzyme on the cell surface were capable of growth using carboxymethyl cellulose as the sole carbon source.

Keywords: Cell surface display, multifunctional cellulolytic enzyme , *E. coli* OmpC

Introduction

Cell surface display allows expression of proteins or peptides on the surface of cells in a stable manner, using the surface proteins of bacteria, yeast, or even mammalian cells as anchoring motifs [3, 4, 8, 13, 19]. Microbial cell surface display is used in vaccine and antibody development, peptide library screening, bioconversion using whole-cell biocatalysis, and bioadsorption [13, 20]. This unrivalled utility of cell surface display has opened new avenues for the development of a variety of applications, ranging from protein library screening to the production of biofuels [25].

Escherichia coli is the most frequently used bacterial host for surface display; as such, a number of *E. coli* display systems that primarily promote the surface exposure of peptides and small proteins have been described [23]. A system for displaying proteins on the surface of *E. coli* was developed using a C-terminal deletion-fusion strategy employing *Salmonella typhimurium* outer membrane protein C (OmpC) as an anchoring motif, providing a new and efficient method for displaying large proteins on the surface of *E. coli* [4].

Cellulose can be degraded to glucose through the synergistic action of three classes of glycoside hydrolases: endo-β-1,4-glucanase, which randomly attacks the cellulose polymer by endoaction; exo-β-1,4-cellobiohydrolases, which remove cellobiose from the non-reducing or reducing ends of the cellulose chain; and β-glucosidase (BGL), which hydrolyzes cello-oligosaccharides and cellobiose to glucose [1, 2, 5]. We recently reported direct screening for endo-type cellulase using carboxymethyl cellulose (CMC) and exo-type cellulase, and a fluorescence-based, robotic, high-throughput screening (HTS) plate assay system [9, 10]. Using this system and a protocol for screening rumen fluid as a metagenomic resource, we identified the novel exocellulase, CelEx-BR12. The CelEx-BR12 cellulolytic

enzyme exhibits characteristics unique to a multifunctional enzyme, displaying endocellulase, exocellulase, and xylanase activities [11].

There have been no reports of a single gene having multifunctional exocellulase activity that, when heterologously expressed on the cell surface of *E. coli*, would allow growth on CMC. Here, we successfully displayed a multifunctional cellulolytic enzyme on the surface of *E. coli*, using *E. coli*-derived OmpC as an anchoring motif. This recombinant *E. coli* expressing a multifunctional cellulolytic single enzyme showed CMC-assimilating activity and exhibited growth using CMC as a carbon source.

Materials and Methods

Materials

CMC and 4-methylumbelliferyl-β-D-cellobioside ($MeUmbG_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest purity that was commercially available. *E. coli* strain MC4100 [F2 *araD139* D*(argF-lac)U169 rpsL150* (Str*) relA1 flbB5301 deoC1 ptsF25 rbsR*] (ATCC 35695) was used as a host strain for all plasmids used in this study. The pTBgl (BGL gene cloned into pTrc99A) plasmid was a kind gift from Dr. Kim (Chonnam National University, Republic of Korea) [7].

Construction of an Expression Vector for the OmpC-Fused CelEx-BR12 Gene in *E. coli*

An optimized gene (*celEx-BR12opti*) [11] encoding CelEx-BR12 (GenBank Accession No. KC963960) was directionally cloned into pTrc99A (Amersham Pharmacia Biotech, Uppsala, Sweden). The glutamine in the second external loop of the C-terminus (Gln_{300}) of *E. coli* K-12 OmpC was selected as the fusion point [26]. OmpC-fused CelEx-BR12 (OCBR12) was obtained by performing overlapping PCR using oligonucleotide primers for truncated OmpC from *E. coli* strain K-12 (OmpC-F, 5′-AGG AAA CAG A CC **ATG** AAA GTT AAA GTA CTG TCC-3′, and OmpC_CBR12-R, 5′-TTC CCG CTT GCT GCA GGT AAG CCA GGG-3′; underlining indicates the region for homologous recombination with the vector, and bold text shows the start codon) and CelEx-BR12 [11] (CBR12_OmpC-F, 5′-TTA CCT GCA GCA AGC GGG AAA GGT CAA TAA-3′, and CBR12-R, 5′-CGA CTC TAG AGG ATC **TTA TTA** ATG GTG ATG ATG GTG ATG TTT CTC TAG GGG CTT TCC T-3′; underlining indicates the region of homology with the vector, and bold text shows the stop codon). Polymerase chain reaction (PCR) using these overlapping primers yielded an approximately 2 kb fragment. The amplified DNA fragment was cloned into the *Nco*I and *Bam*HI site of pTrc99A using an In-Fusion Cloning Kit (Clontech Laboratories, Mountain View, CA, USA) to generate pTOCBR12. The plasmid pTOCBR12-Bgl containing the two enzymes, OmpC-fused CelEx-BR12 and BGL [7], was constructed using the oligonucleotide primers Bgl-F (5′-GCA GGC ATG CAA GCT T**AT G**AT GAT CGA AGC CAA GA-3′; underlining indicates the region of homology with the vector, and bold text shows the start codon) and Bgl-R (5′-CAA AAC AGC CAA GCT T**TT ATC A**TC CCG GCT TGT GGT T-3′; underlining indicates the region of homology with the vector, and bold text shows the stop codon). PCR amplifications were performed with a TProfessional Thermocycler (Biometra, Goettingen, Germany) using pTBgl (BGL gene cloned into pTrc99A) [7] as a template. The amplified DNA fragment was cloned into the *Hin*dIII site of pTOCBR12 using an In-Fusion Cloning Kit (Clontech Laboratories) to generate pTOCBR12-Bgl. *E. coli* XL1-Blue was used as a host strain for the cloning and maintenance of plasmids. The recombinant plasmid was introduced into *E. coli* MC4100 for characterization and recombinant protein production.

Expression of Recombinant OmpC-Fused CelEx-BR12 Enzyme on the Cell Surface

E. coli MC4100 (pTrc99A) and *E. coli* MC4100 (pTOCBR12) were grown in 3 ml of Luria-Bertani (LB) medium containing 50 μg/ml ampicillin at 30°C. Recombinant cells were induced at an optical density at 600 nm (OD_{600}) of 0.4–0.6 by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma Chemical Co.). An outer membrane protein (OMP) fraction was prepared from the induced cultures as a Triton X-100 insoluble fraction, as described by Puente *et al*. [18], and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In brief, the cells were harvested by centrifugation at 7,000 ×*g* for 10 min and washed with ice-cold 10 mM Na_2HPO_4 (pH 7.2). Cells were resuspended in 1 ml of the same buffer and disrupted using a VCX750 sonicator (Sonics Materials Inc., Newtown, CT, USA). Cell debris and intact bacteria were removed by centrifugation for 2 min at 12,000 rpm in a microfuge at room temperature (RT), and the membrane fractions were recovered by microcentrifugation of the supernatant at 12,000 rpm for 30 min at RT. The membrane fractions were resuspended in 500 μl of 10 mM Na_2HPO_4 (pH 7.2) containing 2% (w/v) Triton X-100 and incubated at 37°C for 30 min. The insoluble fraction was recovered by centrifugation at 12,000 rpm for 30 min in a microfuge at 4°C. This insoluble fraction was washed with 10 mM Na_2HPO_4 (pH 7.2) and resuspended in 50 μl of PBS (pH 7.4).

Fluorescence-Activated Cell Sorting

CelEx-BR12 antiserum, produced by immunization of rabbits with purified CelEx-BR12 enzyme, was generated by the custom antibody production services of Youngin Frontier Inc. (Seoul, Republic of Korea). The animal care and experiments performed by Youngin Frontier Inc. were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of Youngin Frontier Inc. The animal handling protocol was in accordance with institutional and international guidelines.

The surface localization of the recombinant OmpC-CelEx-BR12 fusion protein was confirmed by flow cytometry analysis. MC4100 (pTrc99A), used as a negative control, and recombinant cells were harvested and washed three times with PBS, resuspended in PBS

containing 1% bovine serum albumen and rabbit polyclonal anti-CelEx-BR12 antibody (1:25 (v/v)), and incubated at room temperature for 1.5 h. The reacted cells were washed three times with PBS and then incubated with a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. The FITC-labeled cells were examined using a fluorescence-activated cell sorter (FACS; BD Biosciences, San Jose, CA, USA). For each sample, 100,000 clones were randomly analyzed.

Measurement of Exocellulase Activity

The enzyme activity of OMPs prepared from induced recombinant *E. coli* MC4100 (pTOCBR12) and MC4100 (pTrc99A) cells was assayed by measuring the release of the MeUmb group following incubation of an aliquot of the enzyme with 0.1 mM $MeUmbG_2$ in 100 mM sodium acetate buffer (pH 5.0) for 10 min (whole cell) or 30 min (total OMPs) at 37°C, along with the appropriate 4-methylumbelliferone standards (0.05–1 nM). The fluorescence intensity of the released MeUmb groups was determined using a 1420 VICTOR multilabel counter (PerkinElmer Life Sciences, Turku, Finland) using excitation at 365 nm and emission at 460 nm. Assays were performed in 100 mM sodium acetate buffer (pH 5.0) containing 0.1 mM of MeUmb glycoside in a total reaction volume of 100 µl. The reaction was terminated by addition of 100 µl of 500 mM glycine buffer (pH 10.4) [10].

Reusability of the Cell Surface-Displayed Enzyme

Induced recombinant *E. coli* MC4100 (pTOCBR12) cells were harvested by centrifugation at 10,000 ×*g* for 10 min at 4°C. The cells were washed three times and their OD_{600} was adjusted to 1.0 with 100 mM sodium acetate buffer (pH 5.0). Aliquots of washed cells (100 µl) were collected by centrifugation at 10,000 ×*g* for 5 min at 4°C and resuspended at different densities for subsequent assay. The reusability of cells for assaying enzymatic activity was assessed by incubating whole cells in 100 µl of 100 mM sodium acetate buffer (pH 5.0) containing 0.1 mM $MeUmbG_2$ for 10 min at 37°C. The reaction was terminated by centrifugation at 10,000 ×*g* for 5 min at 4°C and addition of 100 µl of 500 mM glycine buffer (pH 10.4). Harvested cells were resuspended in 100 µl of reaction mixture and reused in up to five cycles of subsequent reactions.

Media and Growth Using CMC as a Carbon Source

Cells were subcultured in LB medium supplemented with 50 mg/l ampicillin in a shaking incubator (250 rpm) at 30°C. The cells were induced at an OD_{600} of 0.4–0.6 by adding 1 mM IPTG, after which cell growth was continued for 6 h at 30°C. Cells were inoculated into R/2 medium [17] (2 g/l $(NH_4)_2HPO_4$, 6.75 g/l KH_2PO_4, 0.85 g/l citric acid, 0.7 g/l $MgSO_4 \cdot 7H_2O$, and 5 ml/l trace metal solution consisting of 10 g/l 5 M HCl:$FeSO_4 \cdot 7H_2O$, 2.25 g/l $ZnSO_4 \cdot 7H_2O$, 1 g/l $CuSO_4 \cdot 5H_2O$, 0.35 g/l $MnSO_4 \cdot H_2O$, 0.23 g/l $Na_2B_4O_7 \cdot 10H_2O$, 2 g/l $CaCl_2 \cdot 2H_2O$, and 0.106 g/l $(NH_4)_6MO_7 \cdot O_{24} \cdot 4H_2O$ (pH 6.8)) supplemented with 50 mg/l ampicillin and 0.1% (w/v) glucose, using an aqueous broth suspension of the organism from a previous culture. The basal medium was supplemented with CMC as a carbon source. The CMC was autoclaved separately and added to the R/2 medium to give a final concentration of 1% (w/v).

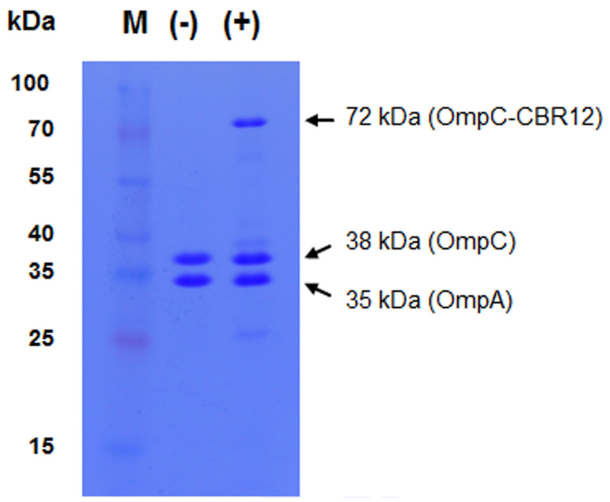

Fig. 1. SDS-PAGE analysis of outer membrane proteins from *E. coli* MC4100 harboring pTrc99A and pTOCBR12.
M, molecular weight marker; (-), *E. coli* MC4100 (pTrc99A); (+), *E. coli* MC4100 (pTOCBR12); OmpC-CBR12, *E. coli* K12 OmpC-fused CelEx-BR12 cellulase; OmpC, *E. coli* MC4100 OmpC; OmpA, *E. coli* MC4100 OmpA.

Results

Expression of Truncated OmpC-Cellulase Fusion Protein in *E. coli* MC4100 (pTOCBR12)

The OmpC-fused CelEx-BR12 gene was amplified and cloned into the pTrc99A vector, as described in Materials and Methods. The resultant plasmid, pTOCBR12, was introduced into *E. coli* MC4100 to yield *E. coli* MC4100 (pTOCBR12). Outer membrane fraction samples were analyzed by SDS-PAGE on 12% (w/v) gels. The estimated molecular mass of OmpC-fused CelEx-BR12 was confirmed to be approximately 72 kDa by SDS-PAGE (Fig. 1). We also confirmed the band using western blot analysis using rabbit polyclonal anti-CelEx-BR12 antibody (data not shown). CelEx-BR12 peptides up to 357 amino acids long (~39.3 kDa) could be targeted efficiently to the *E. coli* outer membrane by the truncated OmpC protein (300 amino acids, ~33 kDa). These results demonstrate the successful display of CelEx-BR12 using truncated OmpC as an anchoring motif.

Confirmation of Multifunctional Cellulase Display on the Cell Surface by FACS Analysis

To confirm the display of multifunctional cellulase on the

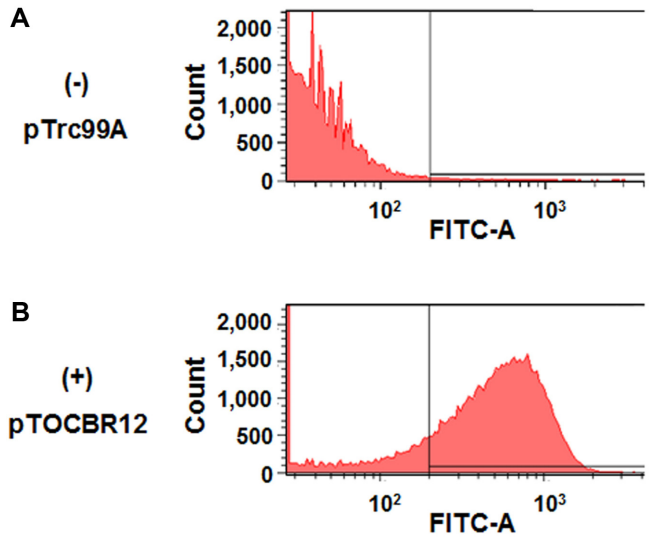

Fig. 2. FACS analysis of recombinant CelEx-BR12-harboring *E. coli* MC4100.
(-), *E. coli* MC4100 (pTrc99A); (+), *E. coli* MC4100 (pTOCBR12).

cell surface, we directly examined recombinant cells by FACS. *E. coli* MC4100 (pTOCBR12) displaying the OmpC-cellulase fusion protein fluoresced owing to the binding of the FITC-conjugated secondary antibody to the anti-CelEx-BR12 primary antibody, indicating successful display of CelEx-BR12 cellulase on the outside of *E. coli* cells (Fig. 2B). In contrast, *E. coli* MC4100 (pTrc99A) cells, which do not express CelEx-BR12, were not fluorescent (Fig. 2A). To confirm the majority of FITC-staining *E. coli* MC4100 (pTOCBR12), we measured exocellulase activity after sorting (data not shown). The results of these functional studies further confirmed the surface localization of the recombinant OmpC-CelEx-BR12 fusion protein in *E. coli* MC4100 (pTOCBR12).

Measurement and Reusability of the Exocellulase Activity from *E. coli* MC4100 Harboring OmpC-Cellulase Fusion Protein

Harvested *E. coli* MC4100 (pTrc99A) and *E. coli* MC4100 (pTOCBR12) cells were washed three times and adjusted to an OD_{600} of 1.0 with 100 mM sodium acetate buffer (pH 5.0) as described in Materials and Methods. Aliquots of washed cells were collected by centrifugation at 11,000 ×*g* for 5 min at 4°C and adjusted to different OD_{600} (up to 5) in the reaction mixture. The enzymatic activity of whole cells was assayed by measuring the release of the MeUmb group upon incubation with a fluorogenic glycoside substrate [10]. Measurements of exocellulase activity of whole cells revealed strong enzymatic activity in *E. coli* MC4100 (pTOCBR12); in contrast, negative control *E. coli* MC4100 (pTrc99A) showed no activity (Fig. 3A), and no exocellulase activity was detected in the medium (data not shown). These results indicate that recombinant *E. coli* displayed active exocellulase on the cell surface and suggest that this enzyme was not secreted into the medium.

We further assessed the function of displayed OmpC-cellulase fusion protein by measuring the exocellulase activity in total OMP fractions prepared from *E. coli* MC4100 (pTrc99A) and *E. coli* MC4100 (pTOCBR12). Incubation of fluorogenic glycoside substrate with different amounts of total OMPs of *E. coli* MC4100 (pTOCBR12) resulted in a strong, dose-dependent increase in fluorescence emission (Fig. 3B), a pattern similar to that produced using intact cells. Like intact *E. coli* MC4100 (pTrc99A), OMPs prepared from these cells showed no exocellulase activity toward the

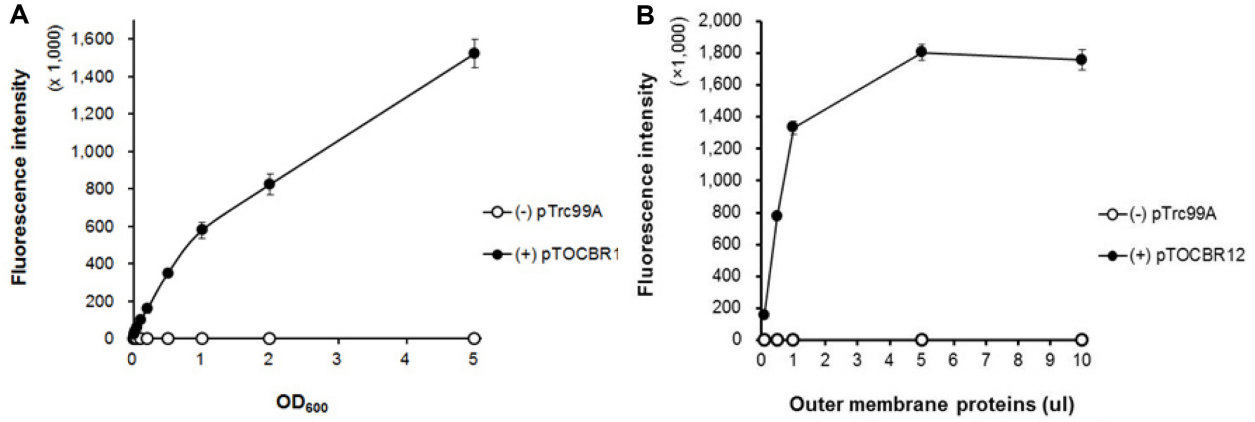

Fig. 3. Exocellulase activity of whole-cell (**A**) and outer membrane (**B**) fractions for *E. coli* MC4100 harboring OmpC-cellulase fusion protein.

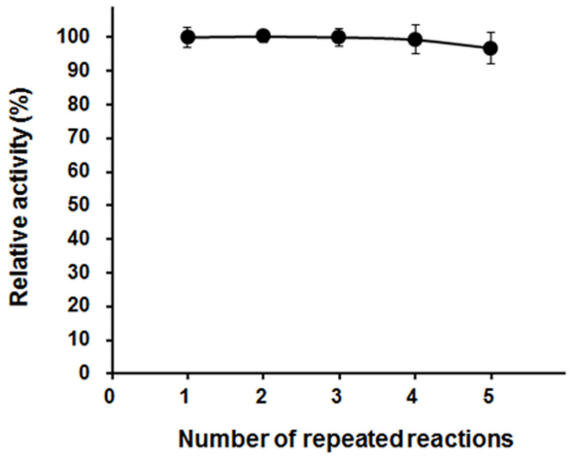

Fig. 4. Time profile of the reusability of *E. coli* MC4100 harboring cell surface-displayed CelEx-BR12 cellulase.

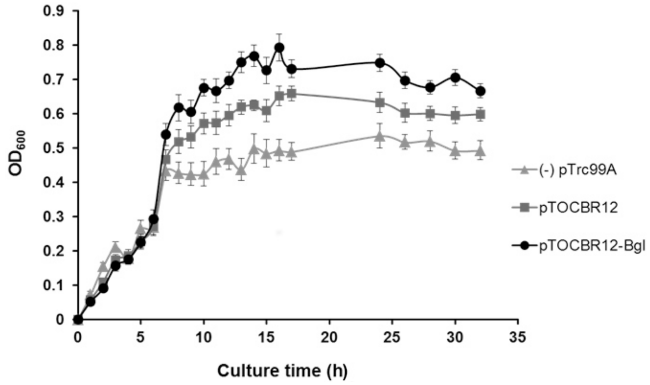

Fig. 5. Synergistic effect of cell surfaced-displayed cellulase on strain growth using CMC as a carbon source.

fluorogenic substrate (Fig. 3B). Collectively, these results confirm the surface localization of recombinant OmpC-CelEx-BR12 fusion protein in *E. coli* MC4100 (pTOCBR12).

The reusability of cell surface-displayed exocellulase was investigated by carrying out repeated reaction cycles with cells using $MeUmbG_2$ as a substrate. As shown in Fig. 4, the catalytic activity of cell surface-displayed exocellulase was maintained for at least five successive reactions, demonstrating the reusability of *E. coli* MC4100 (pTOCBR12) for assaying exocellulase activity. The slight decrease in activity observed was within the error range of individual measurements (93%–100%).

Cell Growth Using CMC as a Carbon Source

To study the ability of recombinant exocellulase-displaying cells to use CMC as a carbon source, we determined the time course of cell growth following inoculation of IPTG (0.1 mM)-induced cells into R/2 medium containing 1% (w/v) CMC, 50 mg/l ampicillin, and 0.1% (w/v) glucose. Unless indicated otherwise, strains harboring pTrc99A, pTOCBR12, and pTOCBR12-Bgl were cultivated at 30°C and were grown for an additional 3 h after induction as described in Materials and Methods. The ability of recombinant cells to use CMC as the sole carbon source was confirmed using R/2 medium containing 1% (w/v) CMC (without glucose) under optimal growth conditions. The growth of *E. coli* MC4100 (pTrc99A), *E. coli* MC4100 (pTOCBR12), and *E. coli* MC4100 (pTOCBR1-Bgl) increased in glucose-free R/2 medium containing 1% (w/v) CMC. *E. coli* MC4100 (pTOCBR12) and *E. coli* MC4100 (pTOCBR1-Bgl) exhibited greater growth compared with *E. coli* MC4100 (pTrc99A) (Fig. 5). The multifunctional cellulase activity of CelEx-BR12 and Bgl enzymes was detectable on the basis of cleavage of internal β-1,4-glucosidic bonds of CMC and hydrolysis of soluble cello-oligosaccharides (*e.g.*, cellobiose) to glucose.

Discussion

Microbial cell surface display has a wide range of biotechnological and industrial applications, including live vaccine development, peptide library screening, bioconversion using whole-cell biocatalyst, and bioadsorption [12, 14]. The main advantage of this technique is that cell surface display allows peptides and proteins to be displayed on the surface of microbial cells by fusing them with the anchoring motifs. Although several variations of the technique have been described, the N-terminal fusion approach in particular is found to be suitable when the carrier protein possesses a directing and anchoring domain in its C-terminus part [13]. In the present study, we used an *E. coli*-derived OmpC as an anchoring motif to successfully display a multifunctional cellulolytic enzyme on the surface of *E. coli*.

Presently, the high cost of production constitutes a major bottleneck for the traditional industrial production of cellulase. On the contrary, cultivating and harvesting recombinant cells are simple processes, and the cost of cell surface-displayed cellulase is much lower than that of purified or immobilized enzyme. Moreover, cell surface-displayed cellulase is stable and thus reusable for repeated rounds of reaction. In addition, the cytotoxicity of enzymes expressed in the cytosol is eliminated, as these enzymes are displayed only on the surface, enabling cell surface display techniques to further improve cellular characteristics.

A review of the literature shows that there is currently no

cell surface display for (only) one cellulase enzyme in *E. coli*. In a cellulosome, multiple enzymes assemble into a macromolecular complex by their association with a scaffold protein for the efficient degradation of cellulose [16]. Several recent studies have reported on the recombinant expression of minicellulosome scaffold proteins in yeast [6, 22]. The design of a cellulosome system is generally based on the use of a surface-bound anchoring scaffoldin, dockerin-tagged adaptor scaffoldins, and dockerin-tagged enzymatic subunits (cellulolytic enzymes) for cellulose hydrolysis on the yeast. This is mainly for the cleavage of accessible ends of cellulose molecules, due to limitations of substrate specificity compared with a cellulosome system [21, 24]. We present here the feasibility of a multifunctional CelEx-BR12 cellulase (*i.e.*, exhibiting endocellulase, exocellulase, and xylanase activities) on the *E. coli* surface, using truncated *E. coli* OmpC as an anchor motif. To date, there have been no reports of heterologous expression in *E. coli* cell surface display of a gene encoding a multifunctional CelEx-BR12 cellulase that would allow growth on CMC. Although *Bacillus thuringiensis* strains have been reported to produce novel cellulases capable of liberating glucose from CMC [15], the use of exocellulase-expressing *E. coli* could lead to a reduction in the use of a general cellulase, significantly reducing the costs of hydrolysis in cellulosic biomass-derived products. It is important to degrade CMC substrates that cannot penetrate into the membrane, by using the successful display of a multifunctional CelEx-BR12 cellulase on the cell surface. Our development of a CMC-assimilating *E. coli* strain through expression of a multifunctional cellulolytic enzyme and demonstration of its ability to grow using CMC as the sole carbon source highlights the applicability of cell surface display techniques for biorefinery applications.

Although cell surface display systems using a multifunctional cellulase in *E. coli* have been developed for various applications, most of these studies have been limited to laboratory research. We believe that this cell surface-displayed cellulase system described here could be used in many fields, including food, biochemical, and medical fields, and environmental industries. However, further studies, including crystallization of the protein for structural validation and potential protein evolution, for multifunctional CelEx-BR12 cellulase are warranted.

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