RESEARCH ARTICLE

Cell Surface Display

Biotechnology Journal

www.biotechnology-journal.com

Development of a Potential Protein Display Platform in *Corynebacterium glutamicum* Using Mycolic Acid Layer Protein, NCgl1337, as an Anchoring Motif

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In the cell surface display, the choice of host cell and anchoring motif are the most crucial for the efficient display of passenger proteins. *Corynebacterium glutamicum* has mycolic acid layer in outer membrane and the use of protein in the mycolic acid layer as an anchoring motif can provide a potential platform for surface display in *C. glutamicum*. All 19 mycolic acid layer proteins of *C. glutamicum* are analyzed, and two proteins, NCgl0535 and NCgl1337, which have a signal peptide and predicted *O*-mycoloylation site, are selected as anchoring motifs candidates. Among them, NCgl1337, which shows better expression with higher display efficiency, is chosen as a potential anchoring motif. Two forms of the NCgl1337 anchoring motif, a full-length (1–324 amino acids) and a short-length (1–50 amino acids) containing only signal peptide and *O*-mycoloylation site, are constructed and their abilities for surface display are examined using two protein models, endoxylanase from *Streptomyces coelicolor* and α-amylase from *Streptococcus bovis*. For both model proteins, the short-length NCgl1337 anchoring motif exhibits higher yield of protein display on the surface of *C. glutamicum* than the full-length NCgl1337. Finally, with *C. glutamicum* displaying α-amylase, a batch fermentation is performed for the production of L-lysine from starch degradation, and a production of L-lysine as high as $10.8 \pm 0.92\ g\ L^{-1}$ was achieved after 18 h of culture.

1. Introduction

Cell surface display, in which recombinant proteins or peptides are immobilized on the surface of bacterial cells, is a powerful tool in various areas of biotechnology, such as bioconversion, bioremediation, live vaccine, protein engineering, and biosensors.[1,2] In most cell surface display platforms, the passenger proteins can be immobilized on the surface via fusion with various carrier proteins (called anchoring motifs), which are natively localized on the exterior structures of host cells. The choice of host cells and anchoring motifs are the most critical for the efficient display of passenger proteins on the cell surface. So far, various microorganisms have been used as hosts for surface display, including Gram-negative and -positive bacteria, and yeast (mainly *Saccharomyces cerevisiae*). Among these, *Escherichia coli* is the most frequently used host because of its capability to produce recombinant proteins at high yields, the availability of various carrier proteins including outer membrane proteins, and the existence of many useful tools for genetic manipulation.[3] However, the complex *E. coli* cell envelope, which is composed of an inner and an outer membrane, can be a big barrier for surface display; additionally, it is not suitable for displaying big and complex proteins. Compared with *E. coli*, Gram-positive bacteria, such as *Bacillus* sp. and *Corynebacterium* sp., would be more suitable hosts for surface display because of their single membrane and thick and rigid cell wall structure; these hosts have been used in several bacterial cell surface display applications.[4–6]

Corynebacterium glutamicum is one of the most useful industrial bacterial strains. Traditionally, it has been widely used for the production of various amino acids including L-lysine, L-glutamate, and L-arginine,[7,8] and recently it has been also used for the production of various biochemical, including succinic acid, lactic acid, cadaverine, putrescine, and γ-aminobutyrate,[9,10] bioethanol,[11,12] recombinant proteins,[13] and pharmaceutical proteins including antibodies, growth factors, and IGF-I.[14–16] As a host for surface display, *C. glutamicum* has additional good characteristics and a few successful platforms have been reported.[17–19] Particularly, *C. glutamicum* is a generally recognized as safe (GRAS) strain; hence, it is suitable for the display of viral antigens, which can be used as oral vaccines.[20,21] For the display of proteins on the surface of *C. glutamicum*, several anchoring motifs have been used, including NCgl1221, NCgl0933 (Porin B, PorB), PorC from *C. glutamicum*, and PgsA of *Bacillus subtilis*.[18,19,22–24] However, similar to most other bacterial hosts, the choice of anchoring motifs is crucial for the display efficiency in *C. glutamicum*. To increase the versatility of the cell surface display technology in *C. glutamicum*, it is necessary to isolate new and potential anchoring motifs.

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DOI: 10.1002/biot.201700509

In this study, we report the development of a novel cell surface display platform in *C. glutamicum* toward a potential whole cell biocatalyst. For this purpose, we isolated a novel anchoring motif from the proteins localized in the mycolic acid layer of *C. glutamicum*. We searched all 19 mycolic acid layer proteins in *C. glutamicum*, and NCgl1337, which has a signal peptide and putative *O*-mycolylation motifs with relatively short length, was chosen as a potential anchoring motif for cell surface display. The suitability of the NCgl1337 for surface display in *C. glutamicum* was examined with two protein models (endoxylanase and α-amylase). Moreover, we examined the production of L-lysine with cells displaying α-amylase to confirm the usefulness of our display platform. Finally, with *C. glutamicum* displaying α-amylase, batch fermentation was performed for the production of L-lysine by degrading starch.

2. Experimental Section

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in **Table 1**. *E. coli* XL1-blue was used as a host for gene manipulation and plasmid maintenance. *C. glutamicum* ATCC 13032 was used for the production of recombinant proteins. Industrial L-lysine producer *C. glutamicum* PKC, kindly provided by the Paik Kwang cooperation (Republic of Korea), was used for the production of L-lysine. *E. coli* was cultured in Luria-Bertani (LB) medium (BD, Franklin Lakes, NJ, USA) at 37 °C. For the culture of *C. glutamicum*, brain heart infusion (BHI) medium (BD, Franklin Lakes, NJ, USA) was used. After overnight culture at 30 °C, the cells were transferred into a 250-mL flask containing 50 mL of fresh BHI medium and were further cultured for 24 h with shaking (200 rpm). For the production of L-lysine in flask cultures, a complex medium containing 50 g soluble starch (Junsei, Tokyo, Japan), 7 g cassamino acid, 15 g yeast extract, 3 g K_2HPO_4, 1 g KH_2PO_4, 2 g urea, 10 g $(NH_4)_2SO_4$, 2 g $MgSO_4$, 200 μg biotin, 5 mg thiamine, 10 mg calcium pantothenate, 10 mg $FeSO_4$, 1 mg $MnSO_4$, 1 mg $ZnSO_4$, 200 μg $CuSO_4$, and 10 mg of $CaCl_2$ in 1 L final volume was used. After 24 h culture, cells were transferred into a 250-mL baffled shake flask containing complex medium (50 mL) at 30 °C with shaking (200 rpm). In all cultures, kanamycin (Km, 25 μg mL^{-1}) was added to the culture medium as a sole antibiotic.

2.2. Bioinformatics Analysis of Protein Sequences

For the identification of the transmembrane helix domain, the protein amino acid sequences were analyzed with the SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP)[25] and the TMHMM v.2.0 software (www.cbs.dtu.dk/services/TMHMM-2.0).[26]

2.3. Plasmid Construction

All enzymes used for gene manipulation were purchased from Enzynomics (Daejeon, Repubilic of Korea). Polymerase chain reaction (PCR) was performed in a C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the Prime STAR HS polymerase (Takara Bio, Inc., Shiga, Japan). All oligonucleotides used for PCR are listed in **Table 2**. For gene expression in *C. glutamicum*, pCES-NMCS with a multiple cloning site (MCS)

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Reference or source
E. coli		
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proABlacIq ZΔM15 Tn10 (Tet^r)]*	Stratagene[a)]
C. glutamicum		
ATCC 13032	Biotin-auxotrophic wild type	ATCC
PKC	Industrial L-lysine producer	Paik Kwang[b)]
Plasmids	Relevant Characteristics	Reference or Source
pCES208	*E. coli* – *C. glutamicum* shuttle vector, Km^r	[50]
pCES-NMCS	pCES208 derivative; MCS and *rrn* terminator, Km	[16]
pCES-RBS	pCES208 derivative; Ribosome binding site, MCS and *rrn* terminator, Km^r	This study
pH36R-NCgl0535	pCES-RBS derivative; NCgl0535 gene, FLAG tag	This study
pH36R-NCgl1337	pCES-RBS derivative; NCgl1337 gene, FLAG tag	This study
pH36R-NCgl1337F	pCES-RBS derivative; full-length NCgl1337 gene	This study
pH36R-NCgl1337S	pCES-RBS derivative; short-length NCgl1337 gene	This study
pH36R-NCgl1337F-XlnA	pCES-RBS derivative; NCgl1337 full gene, endoxylanase gene, FLAG tag	This study
pH36R-NCgl1337S-XlnA	pCES-RBS derivative; NCgl1337 short gene, endxylanase gene, FLAG tag	This study
pH36R-NCgl1337F-Amy	pCES-RBS derivative; NCgl1337 full gene, α-amylase gene, FLAG tag	This study
pH36R-NCgl1337S-Amy	pCES-RBS derivative; NCgl1337 short gene, α-amylase gene, FLAG tag	This study

[a)] New England Biolabs, Beverly, MA, USA. [b)]Paik Kwang industrial Co., LTD., Gunsan, Republic of Korea.

Table 2. List of primers used in the PCR experiments.

Primer name	Sequences (5′ to 3′)[a)]
RBS-F	ATC**TCTAG**ATAACTTTAAGAAGGAGATATACATATGGACTTAC**GCGGCCGC**TGC
RBS-R	GCA**GCGGCCGC**GTAAGTCCATATGTATATCTCCTTCTTAAAGTTA**TCTAGAG**AT
NCgl0535-F	GACCAC**CATATG**GCGAAGAATTCTCGAATCC
NCgl0535-R	GTCA**GCGGCCGC**TTATTTGTCATCGTCATCTTTATAATCGTAGATGTACACCATGTCGC
NCgl1337-F	GTACAC**CATATG**GCTCAGCGAAAACTGG
NCgl1337-R	GTGTCA**GCGGCCGC**TTATTTGTCATCGTCATCTTTATAATCGGCGTTTACTCGATCTCG
NCgl1337F-R	TGACA**GCGGCCGC**TAGAC**ACTAGT**GGCGTTTACTCGATCTCG
NCgl1337S-R	TGACA**GCGGCCGC**TAGAC**ACTAGT**AGCCACACCACCACTTGA
XlnA-F	TGACA**ACTAGT**GCCGAGAGCACGCTCGGCGCCGCGGCGGCGCAGAGCGGTCGCTAC
XlnA-R	GCTCA**GCGGCCGC**TTATTTGTCATCGTCATCTTTATAATCGGTGCGGGTCCAGC
Amy-F	TGACA**ACTAGT**GATGAACAAGTGTCAATGAAAGA
Amy-R	TGAT**GCGGCCGC**TTATTTGTCATCGTCATCTTTATAATCTTTTAGCCCATCTTTATTATAGTTTCCAG

[a)] Restriction enzyme sites and FLAG tags are shown in bold and underline, respectively.

was used as a backbone plasmid. A ribosome-binding site (RBS) was introduced into pCES-NMCS to generate pCES-RBS, as follows. Two synthetic oligonucleotides, RBS-F and RBS-R, were hybridized, digested with *Xba*I and *Not*I, and cloned into pCES-NMCS, yielding pCES-RBS. For the expression of NCgl0535, the NCgl0535 gene was amplified from the chromosomal DNA of *C. glutamicum* ATCC 13032 by PCR with two primers, NCgl0535-F and NCgl0535-R. After digestion with *Nde*I and *Not*I, the PCR product was cloned into pCES-RBS to generate pH36R-NCgl0535. For the expression of NCgl1337, pH36R-NCgl1337 was constructed using the same cloning strategy with a different PCR primer set (NCgl1337-F and NCgl1337-R). For the display of heterologous proteins on the cell surface, two different anchoring motifs (full-length and short-length NCgl1337) were amplified from *C. glutamicum* ATCC 13032 by PCR with two primers sets: i) NCgl1337-F and NCgl1337F-R for full-length NCgl1337 and ii) NCgl1337-F and NCgl1337S-R for short-length NCgl1337. Each PCR product was digested with *Nde*I and *Xba*I. As a model protein, an endoxylanase gene (*xynA*) from *Streptomyces coelicolor* A3(2) [NC_003888.3], was amplified by PCR using the XlnA-F and XlnA-R primers, and the PCR product was digested with *Spe*I and *Not*I. The two PCR products, containing the endoxylanase gene and one of the two anchoring motifs, were ligated and cloned into pCES-RBS yielding pH36R-NCgl1337F-XlnA and pH36R-NCgl1337S-XlnA, in which *xynA* gene was linked to full-length and short-length NCgl1337, respectively. For the display of amylase, the amylase gene (*amyA*) from *Streptococcus bovis* 148 (GenBank No. AB000829.1) was amplified by PCR using the Amy-F and Amy-R primers, and two display systems (pH36R-NCgl1337F-Amy and pH36R-NCgl1337S-Amy) were constructed as described above.

2.4. Protein Fractionation and Analysis

After culture in flasks with shaking, the cells were harvested by centrifugation (6000 rpm, 10 min and 4 °C). The cell pellets were resuspended in phosphate-buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) and disrupted by sonication for 9 min at 50% pulse and 20% amplitude (Sonic, Vibra cell, CT, USA). The total protein fraction was collected after sonication. Following centrifugation of the cell lysates (10 000 rpm for 10 min at 4 °C), the supernatant containing the soluble proteins was collected, and the pellet was resuspended in PBS and stored as the insoluble fraction. The membrane protein fraction was prepared according to a method described previously.[17]

The fractionated protein samples were analyzed by SDS–PAGE and Western blotting. For SDS–PAGE, protein samples were loaded on 12% polyacrylamide gels and following electrophoresis, the gels were incubated in a staining solution (50% (v/v) methanol, 10% (v/v) acetic acid, 1 g L^{-1} Coomassie brilliant blue R-250) for 1 h and destained using a destaining solution (10% (v/v) methanol, 10% (v/v) acetic acid). For Western blotting, proteins resolved by SDS–PAGE were transferred onto a polyvinyl difluoride membrane (Roche, Rotkreuz, Switzerland) for 2 h at 70 mA in a Bio-Rad transblot apparatus (Bio-Rad). The membrane was incubated with a blocking solution (Tris-buffered saline (TBS-T): 24.7 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.5% Tween-20, containing 5 wt.% skim milk) for 1 h at room temperature. The membrane was subsequently incubated with blocking solution containing a horseradish peroxidase (HRP)-conjugated monoclonal anti-FLAG M2 antibody (Sigma–Aldrich, St. Louis, MO, USA) for the immune-detection of FLAG-tagged proteins. After the incubation, the membrane was washed with TBS-T (4 times, 5 min each) and the signal was developed using an ECL kit (GE Healthcare Bio-Science AB, Buckinghamshire, UK).

2.5. Flow Cytometry and Immunofluorescence Microscopy

Cultured *C. glutamicum* ATCC 13032 cells were centrifuged at 6000 rpm for 10 min and were resuspended in PBS so that the

optical density at 600 nm (OD_{600}) was adjusted to 0.1. The resuspended cells were mixed with anti-FLAG M2-FITC antibody (5 μg mL^{-1}) and incubated for 1 h at room temperature. Subsequently, the cells were washed twice with PBS, and the fluorescence intensity was measured using a MoFlo XDP flow cytometer (Beckman Coulter, Inc., Carlsbad, FL, USA). The excitation wavelength was 480 nm and fluorescence of cells was detected with a 530/40 band-pass filter.

For confocal microscopy, the cells were immunostained with anti-FLAG M2-FITC antibody as described above for flow cytometry. Subsequently, they were plated on a poly-L-lysine coated microscopy slide and observed by a confocal microscope (Carl Zeiss LSM510 META, Jena, Germany). The excitation wavelength by an argon laser was 488 nm and images were acquired with a long pass 505 nm filter.

2.6. Endoxylanase Activity Assay

Cultured cells (1 mL) were collected by centrifugation at 6000 rpm for 10 min, and the cell pellets were washed with PBS. Subsequently, the cells were resuspended in 0.2 M potassium phosphate buffer, pH 6.3. The endoxylanase activity was determined according to the 3,5-dinitrosalicylic acid (DNS) method, as described previously.[16] One unit (U) of endoxylanase activity was defined as the amount of enzyme required to liberate 1 μmol of reducing xylose per min, at pH 6.3 and 30 °C.

2.7. α-Amylase Activity Assay

Cultured cells (1 mL) were harvested by centrifugation at 6000 rpm for 10 min. After washing with PBS, the cells were resuspended in 1 mL reaction buffer (0.05 MOPS, 0.2 mM sodium azide, pH 6.9) and diluted by a factor of 10 or 100. Subsequently, 100 μL resuspended cells were mixed with 100 μL of 200 μg mL^{-1} DQ™ starch, labeled with BODIPY® FL dye (Molecular Probes™, Eugene, OR, USA). After incubation in a black tube for 30 min at room temperature, the reaction mixture was centrifuged at 13 000 rpm for 10 min, and the supernatant was added on a black 96-well plate (100 μL/well) (Corning Inc., Corning, NY, USA). Fluorescence was measured by a TECAN Infinite M200 Pro ELISA plate reader (Tecan Group Ltd., Männedorf, Switzerland), with an excitation wavelength of 495 nm and a 515 nm filter. One unit (U) of α-amylase activity was defined as the amount of enzyme required to liberate 1.0 mg of glucose from starch in 3 min, at pH 6.9 and 20 °C.

2.8. Batch Fermentation for L-Lysine Production

As a seed culture, *C. glutamicum* PKC harboring pH36R-NCgl1337S-Amy was inoculated into 50 mL complex medium containing 50 g L^{-1} starch as the sole carbon source, in four 250-mL baffled flasks. After a 24-h culture at 30 °C under shaking (200 rpm), the cells were transferred into 2 L fresh complex medium containing 50 g L^{-1} starch, in a 5 L jar bioreactor (BioCNS, Daejeon, Republic of Korea). The culture temperature was 30 °C. The pH was controlled at pH 7. The DO concentration was maintained at 30% (v/v) by on-line monitoring and automatically increasing the agitation speed up to 1200 rpm. Cell growth was determined by measuring the OD_{600} with a spectrophotometer (Mecasys, Daejeon, Republic of Korea).

2.9. Analysis of Total Sugar and L-Lysine Concentrations

The total sugar concentration in the culture medium was determined according to a modified colorimetric method using a phenol-sulfuric acid reaction.[27] Briefly, 50 μL culture supernatant was mixed with 150 μL H_2SO_4 and 30 μL 5% phenol in water. The mixture was heated for 5 min in a 100 °C heat block. After cooling to room temperature, the mixture was added in a black 96-well plate, and the absorbance at 450 nm was measured with a TECAN Infinite M200 Pro ELISA plate reader.

To determine the L-lysine concentration in the culture medium, the supernatant was analyzed by reversed phase high-pressure liquid chromatography (HPLC, LC-20 AD, CTO-20A, SPD-20A; Shimadzu, Japan) equipped with a Zorbax Eclipse amino acid analysis (AAA) column (4.6 × 150 mm 3.5-Micron; Agilent, Santa Clara, CA, USA). Mobile phase A (10 mM Na_2HPO_4,10 mM $Na_2B_4O_7$, pH 8.2) and mobile phase B (acetonitrile:MeOH:H_2O 45:45:10 by volume) were used for separation of the samples as follows: equilibration (1.9 min, 100% A), gradient elution (16.2 min, 0–57% B; 3.7 min, 57–100% B), and cleaning (4 min, 100% B). Fluoraldehyde-o-phthaldialdehyde (OPA) was used to modify the amino acids in the supernatant. The column temperature was maintained at 40 °C. Samples were treated with OPA and detected with UV at 338 nm.

3. Results

3.1. Identification of a New Anchoring Motif from Proteins in the Mycolic Acid Layer

C. glutamicum is a Gram-positive bacterium, which has a single cytoplasmic membrane with a thick peptidoglycan layer. However, unlike other Gram-positive bacteria, Nocardioform bacteria including *Corynebacterium*, *Nocardia*, *Mycobacterium*, and *Rhodococcus* species, have an additional layer, called the mycolic acid layer, to the outside of the peptidoglycan layer[28,29] (**Figure 1**). The mycolic acid layer consists of mycolic acids (2-branched 3 hydroxyl fatty acids), which are mainly linked to peptidoglycans through the esterification of arabinogalactan.[30,31] It is known that 19 proteins are identified in this mycolic acid layer of *C. glutamicum*,[32] and as they are located in the most exterior structure of *C. glutamicum*, we hypothesized that those proteins could be a potential anchoring motif for surface display (Figure 1). The stability and display efficiency of passenger proteins on the cell surface are highly dependent on the properties of anchoring motif. Thus, it is critical to choose a protein that is localized on the membrane and exhibits high stability.[1,33]

To select the potential anchoring motif among 19 mycolic acid layer proteins, we considered the following three criteria: i) the presence of signal peptide; ii) *O*-mycoloylation motif; and iii)

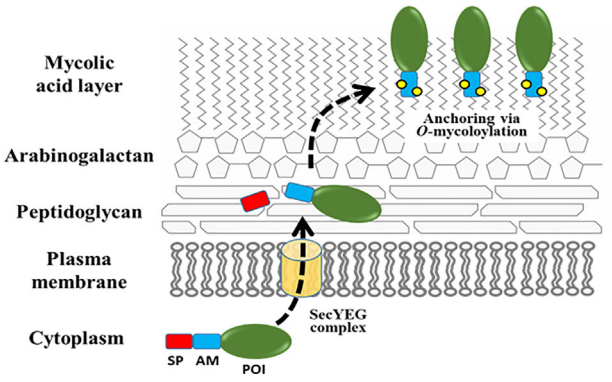

Figure 1. Overal scheme of cell surface display using mycolic acid protein as an anchoring motif in *C. glutamicum*. After translation in cytoplasm, proteins are secreted via Sec-dependent pathway. During protein secretion, signal peptide (SP) is cleaved off and the mature protein is modified by specific posttranslational *O*-mycoloylation on serine residue. Protein of interest (POI) can be anchored on mycomembrane with *O*-mycoloylated anchoring motif (AM). Yellow circles represent *O*-mycoloylation.

protein length. The function of protein is also closely related to the capability of anchoring motif for protein display and needs to be considered as a criteria for selection of candidate. But, unfortunately, only a few proteins in 19 mycolic acid proteins were characterized and most proteins were hypothetical proteins or putative proteins (Table S1, Supporting Information). So, in the present work, we did not consider the function of proteins in the selection of potential anchoring motif. The signal peptide is essential for crossing cytoplasmic membrane and protein display efficiency is highly dependent on the signal peptide characteristics such as length, hydrophobicity, sequence of cleavage site, etc.: total six proteins including NCgl0329, NCgl0776, NCgl0933, NCgl2375, NCgl2430, and NCgl2779, were excluded because it was predicted by SignalP program that those proteins do not have signal peptide. Next, it is recently reported that *O*-mycoloylation of protein is a key post-translational modification for localization of protein on mycolic acid layer.[34,35] Proteins are transported and acylated with one or several mycolic acids by the cMytC on Ser residues located within short linear motifs (SS or SG), and through mycoloylation, protein has enhanced hydrophobicity which is the main driving force for mycomembrane localization. For efficient mycoloylation of proteins, the Ser-rich sequences are preferred, so we also need to consider the presence of Ser-rich sequence in the proteins (particularly in the N-terminus of proteins after cleavage of signal peptide). All five proteins including NCgl0329, NCgl0336, NCgl0381, NCgl0513, and NCgl1048 were excluded because those proteins do not have any Ser-rich sequence. Finally, we also need to consider the protein length for easy handling and efficient display of large proteins: Total six proteins with large size (bigger 40 kDa) including NCgl0987 (43 kDa), NCgl1480 (63 kDa), NCgl2101 (51 kDa), NCgl2777 (70 kDa), and NCgl2789 (71 kDa) were excluded. From these analysis, two mycolic acid layer proteins including NCgl0535 and NCgl1337 were selected. Both NCgl0535 and NCgl1337 contain signal peptides for secretion and predicted *O*-mycoloylation motifs (SS or SG) for anchoring on mycolic acid layer (Figure S1, Supporting Information). In addition, their *O*-mycoloylation motifs are near signal peptide cleavage site, which is beneficial for engineering the length of anchoring motif. To confirm the display of these two proteins on the cell surface, the two plasmids pH36R-NCgl0535 and pH36R-NCgl1337 were constructed for the expression of NCgl0535 and NCgl1337, respectively. In each construct, a FLAG tag (DYKDDDDK) was introduced to the C-terminus of each gene for immunoblotting. Additionally, a strong constitutive promoter (P_{H36})[36] was used to drive gene expression. After cultivation in flasks, protein production and localization were analyzed by SDS–PAGE. We found that both proteins were well expressed and present at the cell surface (membrane fraction) (**Figure 2**A). If the protein are not correctly acylated, proteins cannot be anchored to mycolic acid layer and subsequently are secreted into culture medium.[35] To determine the amount of proteins in extracellular medium, protein samples in culture medium were analyzed by SDS–PAGE. We found that both NCgl1337 and NCgl0535 also existed in extracellular medium and NCgl0535 content was relatively higher than NCgl1337 (Figure S2, Supporting Information).

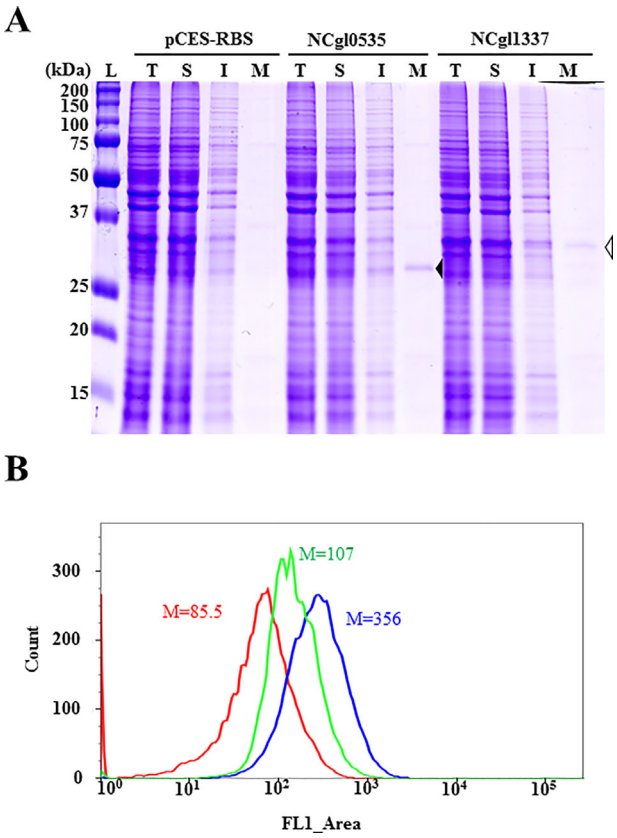

Figure 2. Confirmation of display of mycolic acid layer proteins on cell surface. A) SDS-PAGE analysis of protein production in *C. glutamicum*. Lane L represents molecular weight markers (kDa). Lanes T, S, I, and M represent total, soluble, insoluble, and membrane protein fractions, respectively. The closed and open arrow heads indicate the NCgl0535 and NCgl1337, respectively. B) Flow cytometric analysis. Red, green, and blue histograms represent the fluorescent signal intensities of cells harboring pCES-RBS, pH36R-NCgl0535, and pH36R-NCgl1337, respectively. *M* is mean value of FL1_Area.

The localizations of both proteins were further evaluated by FACS analysis and confocal fluorescence microscopy. In the FACS analysis, cells producing either FLAG tag-fused NCgl1337 or NCgl0535 exhibited higher fluorescence intensities than that of negative control, *C. glutamicum* ATCC 13032 harboring empty plasmid (pCES-RBS) (Figure 2B). Moreover, cells producing NCgl1337 showed higher fluorescent intensity ($M = 356$) than cells expressing NCgl0535 ($M = 107$). These results were also confirmed by confocal fluorescence microscopy. Cells producing either NCgl1337 or NCgl0535 exhibited clear green fluorescence signals, whereas no fluorescence was observed in cells harboring the pCES-RBS plasmid (negative control) (Figure S3, Supporting Information). Taken all results, we concluded that both NCgl1337 and NCgl0535 were successfully localized on the surface of *C. glutamicum*, and NCgl1337 exhibited higher cell surface display efficiency than that of NCgl0535. Taken those results, NCgl1337 was finally chosen as an anchoring motif for the surface display of recombinant proteins on *C. glutamicum*.

3.2. Construction of a Cell Surface Display System Using NCgl1337

NCgl1337 (total of 324 a.a.) has a signal peptide (29 a.a.) and a relatively long extracellular domain (295 a.a.). In general, the surface display efficiency of passenger proteins is highly affected by the length of the anchoring motif.[37,38] Therefore, it was necessary to optimize the anchoring motif length for the efficient display of passenger proteins on the cell surface. For this purpose, we constructed two different surface display systems, pH36R-NCgl1337F and pH36R-NCgl1337S, in which two NCgl1337 constructs of different length were used as anchoring motifs: i) full-length NCgl1337 (1–324 a.a.) and ii) short-length NCgl1337 (1–50 a.a.), including the signal peptide (29 a.a.) and predicted *O*-mycoloylation sites (Figure S4, Supporting Information). The efficiency of protein display in each expression system was evaluated using the following protein models.

3.3. Display of Endoxylanase with the NCgl1337 Anchoring Motif

First, we examined the display of endoxylanase from *Streptomyces coelicolor* A3(2), which can degrade hemicellulose (xylan) to xylooligomers.[36] Cells displaying endoxylanase on their surface can be a useful whole-cell catalyst for the bioconversion of hemicellulose.[39,40] For the surface display of endoxylanase, two plasmids (pH36R-NCgl1337F-XlnA and pH36R-NCgl1337S-XlnA) were constructed using the two anchoring motifs: full-length and short-length NCgl1337. After cultivation, the production and display of endoxylanase in each platform were analyzed. In SDS–PAGE analysis, it was confirmed that endoxylanase was well expressed and successfully localized at the membrane in both platforms (**Figure 3**A). The use of the short anchoring motif (pH36R-NCgl1337S-XlnA), exhibited relatively higher expression levels and localization on the membrane than the full-length NCgl1337 (pH36R-NCgl1337F-XlnA). To confirm endoxylanase display on the cell surface, the

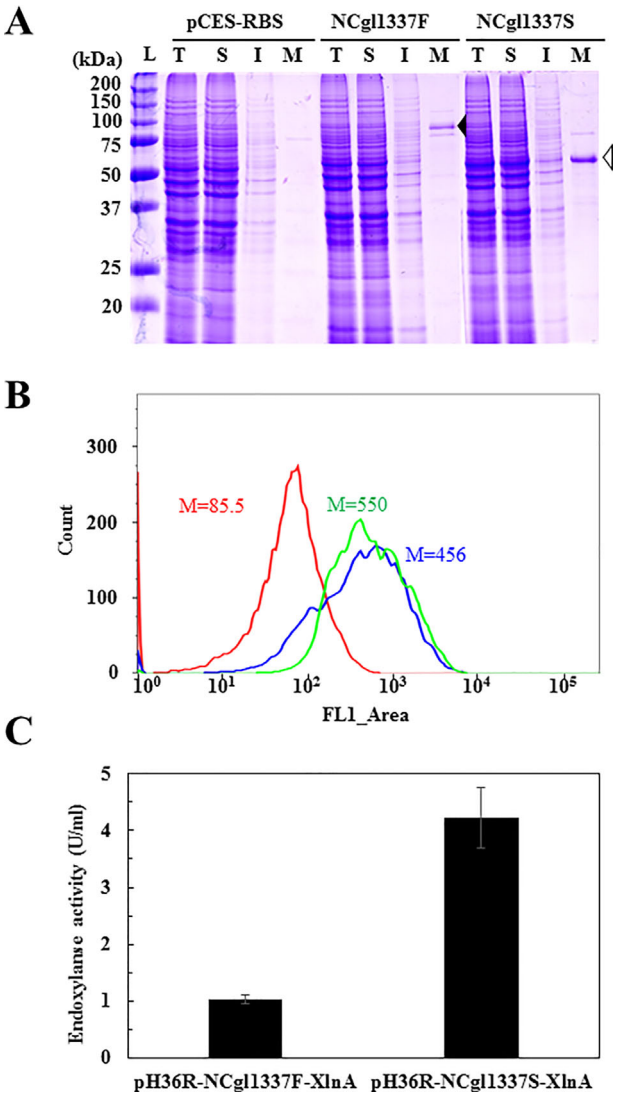

Figure 3. Display of endoxylanase on the cell surface using two forms of NCgl1337 as anchoring motifs. A) SDS–PAGE analysis of endoxylanase expression. Lane L represents molecular weight markers (kDa). Lanes T, S, I, and M represent total, soluble, insoluble, and membrane protein fractions, respectively. The closed and open arrowheads indicate the endoxylanase with full-form (NCgl1337F, 81 kDa) or short-form (NCgl1337S, 52 kDa) of NCgl1337, respectively. B) Flow cytometric analysis. Red, blue, and green histograms represent cells harboring pCES-RBS, pH36R-NCgl1337F-XlnA, and pH36R-NCgl1337S-XlnA, respectively. *M* is mean value of FL1_Area. C) Endoxylanase activity assay with *C. glutamicum* harboring either pH36R-NCgl1337F-XlnA or pH36R-NCgl1337S-XlnA.

cells were labelled with a FITC-conjugated anti-FLAG antibody, and then their fluorescence was analyzed by flow cytometry. It was clearly observed that all cells exhibited fluorescence signals higher than the negative control and cells displaying endoxylanase with the short-length NCgl1337 also exhibited higher intensity than the cells displaying endoxylanase with the full-length form (Figure 3B). To confirm the whole cell catalyst activity, we examined the activity of xylan degradation with both displaying cells. Similar to the earlier results, *C. glutamicum* with

the short-length NCgl1337 resulted in approximately fourfold higher activity (4.22 ± 0.53 U mL^{-1}) than cells with the full-length NCgl1337 (1.03 ± 0.07 U mL^{-1}) (Figure 3C).

3.4. Display of α-Amylase with NCgl1337 Anchoring Motifs

As another display model, we examined the α-amylase from *Streptococcus bovis* 148, which can degrade starch to glucose,[16] and whose display on bacterial cell surfaces can be used for the development of whole cell biocatalysts for the bioconversion of the starch biomass.[41] We also constructed the two display platforms with full and short-length NCgl1337 (pH36R-NCgl1337F-Amy and pH36R-NCgl1337S-Amy), and after flask cultivation, the production and display of amylase in each system were compared. Similar to the earlier results with endoxylanase, higher-level of α-amylase display was achieved by using the short-form (82 kDa) than with the full-length form (111 kDa) (**Figure 4**A). In the FACS analysis, cells displaying amylase with short-length NCgl1337 also exhibited much higher fluorescence intensity ($M = 1010$) than cells with full-length ($M = 341$) (Figure 4B). In the amylase activity assay, cells displaying amylase with short-length NCgl1337 also exhibited 14-fold higher activity (50.8 ± 5.2 U mL^{-1}) than cells displaying amylase with full-length NCgl1337 (3.58 ± 1.2 U mL^{-1}) (Figure 4C).

3.5. Production of L-Lysine from Starch Using *C. glutamicum* Displaying α-Amylase

To demonstrate the usefulness of NCgl1337-based surface display, we conducted flask cultivation for the production of L-lysine from starch using *C. glutamicum* displaying α-amylase. L-lysine producing *C. glutamicum* PKC harboring either pCES-RBS, pH36R-NCgl1337F-Amy, or pH36R-NCgl1337S-Amy were cultivated in media containing starch (50 g L^{-1}) as a sole carbon source and, cell growth and L-lysine production were analyzed. As a negative control, cells harboring pCES-RBS could not grow well in starch medium because of their disability to utilize starch, and L-lysine production was consequently very low (**Figure 5**A). In contrast, cells harboring either pH36R-NCgl1337F-Amy or pH36R-NCgl1337S-Amy grew much better in the starch media, up to 10 of OD_{600} (Figure 5A). Cells with the short form (pH36R-NCgl1337S-Amy) exhibited much higher sugar hydrolysis (up to 90%) and L-lysine production at 60 h (4.39 ± 0.20 g L^{-1}), which was 2.5-fold higher than that (1.75 ± 0.04 g L^{-1}) of cells with full-length form (Figure 5B and C).

Next, for the production of L-lysine from starch in large scale, we performed batch fermentation in a 2-L bioreactor with *C. glutamicum* (pH36R-NCgl1337S-Amy) displaying amylase with short-length NCgl1337. Cells were cultivated in starch (50 g L^{-1})-containing medium, and starch hydrolysis and lysine production were monitored. During the cultivation, 37.2 ± 0.082 g of starch was hydrolysed (74.4%) and cells grew up to 37.9 of OD_{600} in 18 h and went to stationary growth phase (**Figure 6**). From the beginning of cultivation, L-lysine concentration increase continuously and maximum titer (10.8 ± 0.92 g L^{-1}) could be achieved at 18 h.

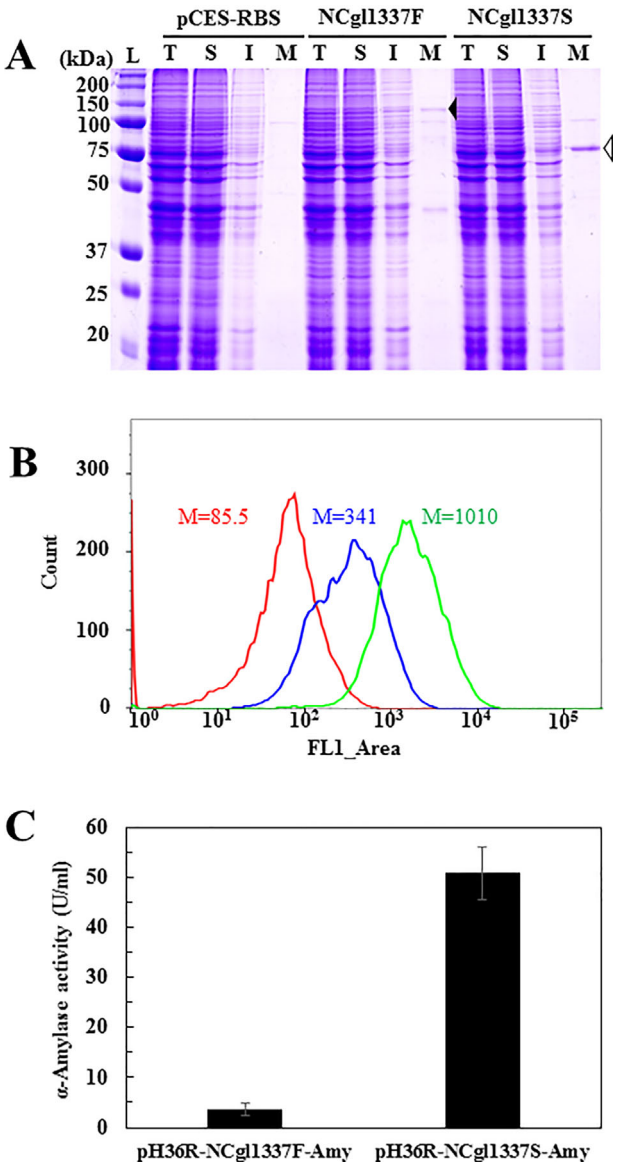

Figure 4. Display of α-amylase on the cell surface using two forms of NCgl1337 as anchoring motifs. A) SDS–PAGE analysis of α-amylase expression. Lane L represents molecular weight markers (kDa). Lanes T, S, I, and M represent total, soluble, insoluble, and membrane protein fractions, respectively. The closed and open arrowheads indicate the α-amylase with full-form (NCgl1337F, 111 kDa) or short-form (NCgl1337S, 82 kDa) of NCgl1337, respectively. B) Flow cytometric analysis. Red, blue, and green histograms represent cells harboring pCES-RBS, pH36R-NCgl1337F-Amy, and pH36R-NCgl1337S-Amy, respectively. *M* is mean value of FL1_Area. C) α-Amylase activity assay with *C. glutamicum* ATCC 13032 harboring either pH36R-NCgl1337F-Amy or pH36R-NCgl1337S-Amy.

4. Discussion

For the development of cell surface display systems, the choice of anchoring motif is the most important issue, and it is closely related to the display efficiency and activity of the passenger proteins. In the present work, we isolated the NCgl1337 as a potential anchoring motif, which is natively localized on the

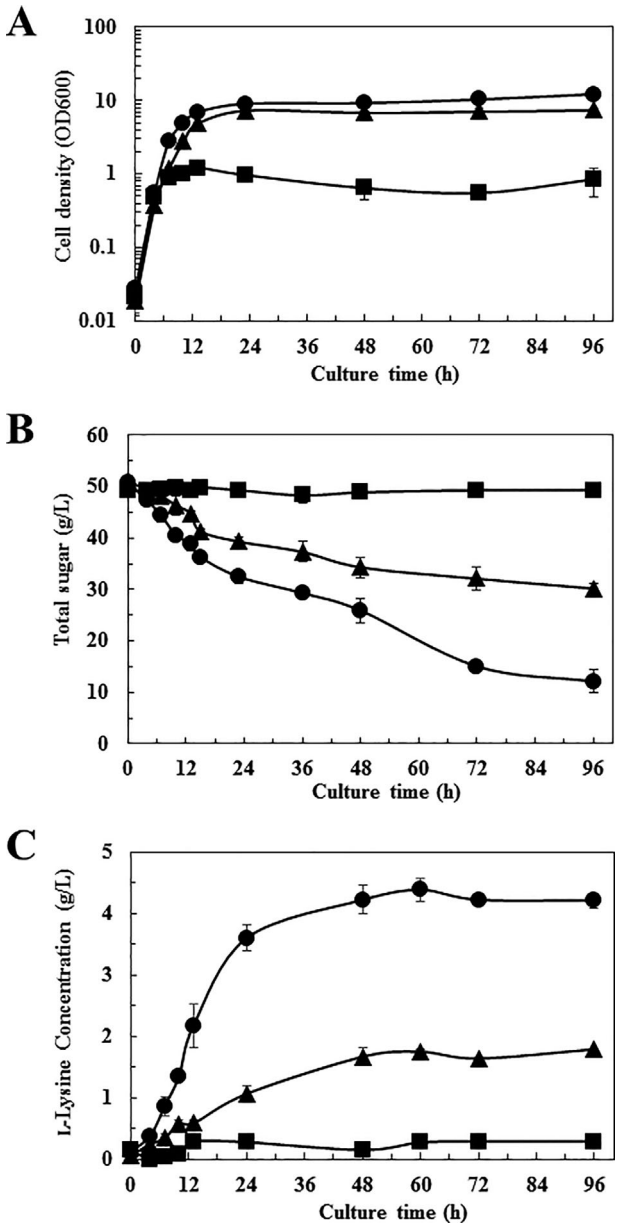

Figure 5. Time profiles of cell growth, sugar consumption, and L-lysine production during cultivation of L-lysine producer *C. glutamicum* PKC displaying α-amylase on cell surface. A) Time profiles of cell growth (OD_{600}). B) Time profiles of total sugar ($g\,L^{-1}$). C) Time profiles of L-lysine concentration ($g\,L^{-1}$). Symbols: Squares (■), cell harboring pCES-RBS; Triangles (▲), cell harboring pH36R-NCgl1337F-Amy; Circles (●), cell harboring pH36R-NCgl1337S-Amy. The data points represent the mean value and standard deviation of biological triplicate.

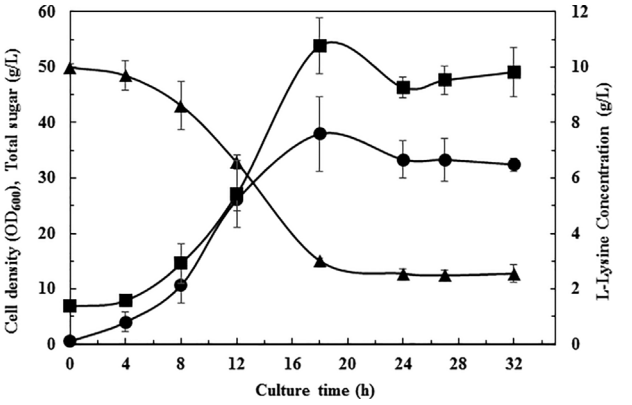

Figure 6. Time profiles of cell growth, sugar consumption, and L-lysine production during batch cultivation of *C. glutamicum* PKC harboring pH36R-NCgl1337S-Amy. Symbols: Circles (●), cell concentration (OD_{600}); Triangles (▲), concentration of total sugar ($g\,L^{-1}$); Squares (■), concentration of L-lysine ($g\,L^{-1}$). The data points represent the mean value and standard deviation of biological duplicate.

mycolic acid layer. NCgl1337 has a serine-glycine-asparagine-histidine (SGNH) hydrolase domain, which acts as an esterase and lipase, and is important for cell wall synthesis and metabolism.[42,43] NCgl1337 have a signal peptide (29 a.a.) following predicted *O*-mycoloylation site (SS or SG), and it was clearly demonstrated that the use of a short form including the signal peptide and *O*-mycoloylation site could provide a potential platform for protein display on the surface of *C. glutamicum*. Previously, several proteins including PgsA, PorB, PorC, PorH, and NCgl1221 were developed as anchoring motifs for cell surface display in *C. glutamicum*.[17–19] Among them, PorB, PorC, and PorH are localized in the mycolic acid layer, similar to NCgl1337,[18] and all are porin proteins, which function in the uptake and passage of small molecules through the mycolic acid layer.[44,45] However, these porins form channel complexes (e.g., PorB forms a pentameric complex),[45] and this might hinder the transport of passenger proteins (particularly big and complex proteins) to the surface. In contrast, using short-length NCgl1337 has a compact structure, and this induces less hindrance for cell surface display. Recombinant proteins could be easily displayed by adding just 50 amino acids. Indeed, the use of the short-length NCgl1337 for the display of α-amylase resulted in at least ninefold higher display ($50.8 \pm 5.2\,U\,mL^{-1}$) of α-amylase compared with those of other display systems employing PorH ($4.9\,U\,mL^{-1}$), PorC ($4.7\,U\,mL^{-1}$), and NCgl1221 ($0.0828 \pm 5.2\,U\,mL^{-1}$) as anchoring motifs.[18,19] Regarding of the higher activity of short-length NCgl1337 than full-length form (Figures 3C and 4C), the possible change of protein conformation by fusion with different anchoring motifs (short or full-length NCgl1337) can be considered, which change can give an effect on the specific activity of proteins. Although we do not have experimental data on the specific activity of displayed proteins, it is supposed that the fusion with NCgl1337 anchoring motif did not give any profound effect on the conformation and specific activity of the displayed proteins. As shown in Figures 3 and 4, the use of short-length NCgl1337 allowed much higher display of fused proteins (endoxylanase and α-amylase) than those of full-length form, and similarly high activities were also obtained using short-form, which implies that the higher activity with short-from was attributed to the higher expression not to conformational change by fusion.

The usefulness of the NCgl1337-based display system for large-scale fermentations was also successfully demonstrated. *C. glutamicum* displaying α-amylase hydrolyzed starch that was supplied as a sole carbon source, and as much as $10.8\,g\,L^{-1}$ of L-lysine could be produced, a value higher than that previously

reported (6.04 g L^{-1}) with a PgsA anchoring motif.[19] The successful production of L-lysine from starch hydrolysis by cells displaying α-amylase also indicates that our system could be applied for the development of consolidated bioprocess (CBP). CBPs featuring i) enzymatic hydrolysis of biomass for cell to utilize sugars and ii) microbial fermentation for the production of target products in single bioreactors have been suggested as an ideal process with outstanding potential for lower costs and higher efficiency.[46,47] In addition to α-amylase for starch degradation, other biomass-degrading enzymes, such as endoxylanase for hemicellulose degradation, could be employed in our NCgl1337-based display platform, and a new CBP for the production of useful products from hemicellulose (xylan) could be developed. From the point of substrates (i.e., starch, cellulose, and hemicellulose) accessibility, the use of long-length anchoring motif may be more efficient compared with short-length anchoring motif. However, it does not always guarantee high efficiency of cell surface display to use of long anchoring motifs.[48,49] Also, we need to consider the content of the displayed protein on the surface. As shown here (Figures 3–5), the use of short-length NCgl1337 allowed much higher expression and secretion efficiency, and consequently much higher activity can be achieved. This can be more critical when the much bigger or complex proteins are displayed.

In addition to NCgl1337, we also chose NCgl0535 as an anchoring motif candidate, but it showed lower efficiency than NCgl1337 (Figure 2). The lower efficiency of NCgl0535 might be ascribed to the less *O*-mycoloylation sites: it is predicted that NCgl1337 has five sites (Ser-rich or Ser-Gly sequences) for *O*-mycoloylation, while only two sites were predicted in NCgl0535 (Figure S1, Supporting Information). The less *O*-mycoloylation sites in NCgl0535 might result in higher generation of non-acylated protein and those proteins were not anchored but secreted into culture medium as shown in Figure S2, Supporting Information. Although we did not examine the short-version of NCgl0535, it is also supposed that the display efficiency of NCgl0535 may not be improved due to the less *O*-mycoloylation sites compared with short-length NCgl1337.

In conclusion, we successfully isolated the potential anchoring motif NCgl1337 from mycolic acid layer proteins, and developed an efficient surface display platform with C-terminal truncated NCgl1337 in *C. glutamicum*. By anchoring the passenger proteins on the most exterior structures (mycolic acid layer) in *C. glutamicum*, the displayed proteins could react with their substrates more efficiently. The usefulness of the NCgl1337-based surface display system was successfully demonstrated with two industrial enzymes, endoxylanase, and α-amylase. We also demonstrated the cell growth and L-lysine production by the hydrolysis of starch in the media, indicating that our display platform has high potential as a whole biocatalyst in the bioindustry.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the Intelligent Synthetic Biology Center of Global Frontier Project (Grant no. 2014M3A6A8066443) and by the National Research Foundation of Korea (Grant no. NRF-2015R1A2A2A01007674) funded by the Ministry of Science, ICT and Future Planning (MSIP).

Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

Corynebacterium glutamicum, cell surface display, mycolic acid, NCgl1337

Received: July 30, 2017
Revised: September 26, 2017
Published online: November 15, 2017

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