A novel cell-surface display system for heterologous gene expression in *Escherichia coli* by using NCgl1221\(^{423}\) as the anchoring protein

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We developed a novel *Escherichia coli* cell surface display system by employing the NCgl1221 protein truncated at AA 423 (for example NCgl1221\(^{423}\)) as an anchoring motif. The enzyme selected for display was α-amylase (AmyA) from *Streptococcus bovis* 148. The N terminus of AmyA was fused to NCgl1221\(^{423}\), and the resulting fusion protein was expressed on the cell surface by IPTG induction. The NCgl1221\(^{423}\)-AmyA fusion protein was shown to be displayed by immunofluorescence microscopy and flow cytometric analysis, while AmyA without fusion to the C terminus of NCgl1221\(^{423}\) anchor was only expressed in the cytoplasm. This result indicated that NCgl1221\(^{423}\) from *Corynebacterium glutamicum* contains a robust surface anchor to attach large target proteins toward the cellular membrane of *E. coli*. The displayed enzyme was active form, and AmyA activity on the cell surface reached 48.7 ± 1.6 U/l after 8 h of induction, while no activity was detected in control cells. The activity of displayed AmyA was increased during cultivation and reached the maximum value of 88.3 ± 2.4 U/l at 16 h of induction. Our results provided the successful application of NCgl1221\(^{423}\) anchor in the *E. coli* cell surface display system.

Key words: α-Amylase, cell-surface display, *Corynebacterium glutamicum*, NCgl1221.

INTRODUCTION

The development of displaying and anchoring heterologous proteins on the surface of bacterial cells has greatly progressed since the bacterial surface display system was first reported in 1986 by Freudi et al.. Different types of surface display systems have been exploited and developed for Gram-positive and Gram-negative bacteria, notably *Escherichia coli* (Samuelson et al., 2002; van Bloois et al., 2011). *E. coli* is the most frequently used host because of its well studied knowledge of genetic and biomolecular systems which makes it useful for efficient transformation and high recombinant protein production. A large number of carrier proteins have been described for displaying different passengers on the surface of *E. coli* (van Bloois et al., 2011). The size limitation of the target protein is a significant problem in the current *E. coli* cell surface display systems. A recent study showed that the largest target protein P450 BM3 with a molecular size of 119 kDa was successfully displayed on the *E. coli* cell surface based on ice nucleation protein (INP) system (Yim et al., 2010). However, outer membrane proteins were the most commonly utilized motifs anchoring heterologous proteins with large molecular sizes on the cell surface of *E. coli*. The outer membrane proteins Omp1 and the C-terminally truncated variant of OmpC were developed as the anchoring proteins to display passengers of 56 kDa and 50 kDa, respectively (Xu and Lee, 1999; He et al., 2008; Baek et al., 2010). Furthermore, the truncated OprF and the transmembrane protein PgsA were also used as...
the anchoring motifs to display large proteins on the surface of *E. coli* cells (Lee et al., 2005; Narita et al., 2006).

The NCGl1221 protein from *Corynebacterium glutamicum* has been proved to be a transmembrane protein with its function of L-glutamate transportation by structural and functional analysis (Nakamura et al., 2007; Yao et al., 2009). It has been reported that the NCGl1221 protein with four transmembrane segments can be located in the cytoplasmic membrane of both *C. glutamicum* and *E. coli* cells (Yao et al., 2009). The NCGl1221 protein truncated at AA 423 (for example, NCGl1221_{423}), one of the mutants of NCGl1221, can constitutively transport glutamate without any alterations of membrane tension (Nakamura et al., 2007). It was suggested that the transmembrane structure and the transport function of NCGl1221 are not affected by the C-terminally truncated mutation. Thus, we can speculate that the NCGl1221_{423} protein probably contains a robust surface anchor which is one of the requirements for the cell surface display.

In this study, we described the successful application of the NCGl1221_{423} protein in the *E. coli* cell surface display system. As the heterologous target enzyme we selected β-amylose (AmyA) from *Streptococcus bovis* 148 described previously (Satoh et al., 1993). We succeeded in showing that, through its C terminal fusion, the NCGl1221_{423} protein can be utilized as the anchoring motif in *E. coli* to display large passengers with a conformationally active form. This is the report of a novel approach for engineering *E. coli* to display an enzyme of more than 75 kDa on the cell surface.

### MATERIALS AND METHODS

#### Bacterial strains and growth conditions

*E. coli* DH5α [supE44 ΔlacU169 (φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] (Invitrogen, Carlsbad, Calif.) was used for recombinant DNA manipulation. *E. coli* strain BL21 (DE3) [F' ompT hsdS F (rha mha) gal dcm (DE3)] (Novagen, EMD Biosciences, Inc., USA) was used as a host for gene expression and cell surface display. Cells were cultured in Luria-Bertani (LB) medium (10 g of tryptone/liter, 5 g of yeast extract/liter, and 5 g of NaCl/liter) or on LB agar plates at 37°C. When necessary, kanamycin was added at a final concentration of 50 mg l\(^{-1}\). *C. glutamicum* ATCC 13032 was cultivated aerobically at 30°C with LB medium (Eggeling and Bott, 2005).

### Construction of recombinant plasmids for cell-surface display

All enzymes for molecular biology, including Ex-Taq polymerase and restriction enzymes, were from TAKARA biotechnology Co. Ltd. (Dalian, China). Restriction digestion, ligation, agarose gel electrophoresis and other DNA manipulations were carried out according to standard procedures (Sambrook and Russell, 2001).

PCR primers used to construct the plasmids are listed in Table 1. The genome DNA of *C. glutamicum* ATCC 13032 was extracted using a genomic DNA Extraction Kit (Promega, Madison, WI, USA). The NCGl1221_{423} protein was amplified by PCR with the primer pair 1 and 2 using the chromosomal DNA of ATCC 13032 as a template. The linker sequence (Gly\(_{2}\)Ser\(_{2}\)) between the NCGl1221_{423} anchor and α-amylose (AmyA) was introduced by primer 2 (Cao et al., 2006). The restriction sites (Table 1) were added to NCGl1221_{423} by the primer pair 1 and 2a. The resulting PCR product purified by QiAQuick PCR purification Kit (Qiagen, Düsseldorf, Germany) was digested with *NdeI* and *BamHII*, and inserted into similarly double digested PET-28a to create PET-N. The DNA fragment encoding the mature AmyA fused with the FLAG tag (DYKDDDDK) was amplified by PCR with the primer pair 3 and 4 from the plasmid pNAT. The restriction sites (Table 1) were introduced at the 5’ and 3’ terminus of the fragment. Subsequently, the resulting fragment excised by *BamHII* and *HindIII* was moved into similarly double digested plasmids PET-28a and PET-N to create PET-A and PET-NA, respectively. The recombinant plasmids were identified by agarose gel electrophoresis.

### Preparation of subcellular fractionation of *E. coli*

Plasmids PET-28a, PET-A and PET-NA were, respectively, transformed into competent *E. coli* BL21(DE3). The kanamycin-resistant transformants were selected and induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 h of continuous shaking. The recombinant cells were harvested at 4°C by centrifugation at 3,500 x g for 10 min, and then washed twice with 1 ml of 10 mM Na\(_2\)HPO\(_4\) buffer (pH 7.2). The final cell pellet was resuspended in 0.5 ml 10 mM Na\(_2\)HPO\(_4\) buffer (pH 7.2) and disrupted by three cycles of sonication on ice for a 10 s-pulse with an intervening 10 s-pause until the cells were completely lysed. The lysate was centrifuged at 12,000 x g for 3 min at 4°C. Then the supernatant was centrifuged at 12,000 x g for 30 min at 4°C to separate membrane proteins. The cytoplasmic sample was obtained as the supernatant and the pellet was resuspended in 0.5 ml 10 mM Na\(_2\)HPO\(_4\) buffer (pH 7.2) containing 0.5% sarcosyl (Lee et al., 2004). The membrane proteins were obtained by centrifugation at 12,000 x g for 30 min at 4°C after incubation at 37°C for 30 min. The pellet containing membrane proteins was washed with 1 ml Na\(_2\)HPO\(_4\) buffer and resuspended in 50 μl of Tris/EDTA buffer (pH 8.0).

### Table 1. Primers used for construction of recombinant plasmids

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>5’-GGTGTCCATATGCGTATTATCAGACGGAGTG-3’</td>
<td><em>NdeI</em></td>
</tr>
<tr>
<td>Primer 2</td>
<td>5’-GCTGCCACCTCCACCCTACGCGCCGCTCCCACCCTAGTGGGCACGTATC-3’</td>
<td><em>BamHII</em></td>
</tr>
<tr>
<td>Primer 2a</td>
<td>5’-GTTTGGATCCCTCAAAGCCTGACCTCCAAAGCCGTGAC-3’</td>
<td><em>BamHII</em></td>
</tr>
<tr>
<td>Primer 3</td>
<td>5’-GTTGCAAGCTTCTGTATCCTGGGACCTGATC-3’</td>
<td><em>HindIII</em></td>
</tr>
<tr>
<td>Primer 4</td>
<td>5’-GTTGTAAGCCTATTGTCATCAGCTTAGCTTGG-3’</td>
<td><em>HindIII</em></td>
</tr>
</tbody>
</table>
Western blot analysis

The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% gel and then blotted to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked for 2 h at 37°C with tris-buffered saline Tween-20 (TBST) (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20, pH 8.0) containing 5% skimmed milk. After washed with TBS, the membrane was allowed to react with primary mouse anti-FLAG M2 (Sigma) and secondary goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody. After washing, TMB (Promega) was finally added to the corresponding reaction for color development.

Immunofluorescence microscopy

The recombinant E. coli cells induced by IPTG were harvested by centrifugation at 3,500 × g for 5 min at 4°C and washed with phosphate-buffered saline (PBS) solution. The cell pellet was resuspended in PBS solution supplemented with 10 g/l bovine serum albumin (Sigma) and then incubated at room temperature for 30 min. Subsequently, the cells were incubated with the primary antibody (mouse IgG against FLAG at a dilution of 1:500) in PBS solution containing 10 g/l bovine serum albumin for 1.5 h at room temperature. After washing five times with PBS solution, the cells were incubated for 1 h at room temperature with goat anti-mouse IgG (H + L) conjugated with Alexa Fluor 488 at a dilution of 1:500. After washing five times with PBS, the cells were observed by microscopy. Immunofluorescence microscopy analysis was performed according to our previous studies (Yao et al., 2009).

Flow-cytometric analysis

The recombinant E. coli cells were immunostained as described above. Flow-cytometric analysis was performed as described previously on a FACSCalibur flow cytometer (BD biosciences) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a cell-sorting catcher tube (Tateno et al., 2007).

Measurement of α-amylase (AmyA) activity

AmyA activity was measured with an α-amylase measurement kit (Kikkoman, Tokyo, Japan) using 2-chloro-4-nitrophenyl 6'-azido-6'-deoxy-β-maltopentaoside (N3-G5-β-CNP) as the substrate. Activity of subcellular fractions from different cells was measured as follows: 40 μl of fraction samples and 400 μl of reaction solution were mixed to prepare the assay mixture. Then the mixture was incubated at 37°C for 10 min, and the reaction was terminated by adding 800 μl of a reaction stop solution. AmyA activity was determined by measuring the absorbance of the liberated 2-chloro-4-nitrophenol (CNP) at 400 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1 μmol CNP per minute from N3-G5-β-CNP at 37°C. Activity of AmyA displayed on the E. coli cell surface was measured in this way. To prepare the sample solution, the recombinant E. coli cells collected from the culture were washed and resuspended in PBS (pH 7.2) by vigorous mixing (Narita et al., 2006; Tateno et al., 2007).

RESULTS AND DISCUSSION

Expression of the fusion protein on E. coli cell surface

Previous studies suggested that the NCgl1221423 protein contains a robust surface anchor which can be used to display target proteins at the cell surface (Nakamura et al., 2007; Yao et al., 2009). However, E. coli is the most attractive host for cell surface display and a variety of E. coli display systems have been reported recently (van Bloois et al., 2011). Herein, we described the application of the NCgl1221423 anchor protein in the E. coli cell surface display system. The plasmid PET-NA for expression of the NCgl1221423-AmyA fusion protein and PET-A for expression of AmyA were constructed and transformed into E. coli BL21 (DE3). Since the recombinant proteins have a FLAG tag at the C terminus of AmyA, the expressed recombinant proteins induced by IPTG in different subcellular fractions were analyzed by SDS-PAGE and Western blotting with mouse anti-FLAG M2 antibody. As shown in Figure 1A, the recombinant E. coli cells harboring the plasmid PET-NA produced the NCgl1221423-AmyA fusion protein in the membrane fraction at a high level after 8 h of IPTG induction, while no corresponding proteins were produced in the membrane fractions of the E. coli cells harboring the empty PET-28a and PET-A. The molecular sizes of NCgl1221423 with the linker (Gly4Ser)2 at its C terminus and the AmyA-FLAG protein were approximately 49 and 78 kDa, respectively, and that of the fusion protein was therefore approximately 127 kDa. The clear band of the fusion protein was observed at the estimated molecular size in Western blot analysis (see in Figure 1B), indicating the successful expression of the fusion protein in the membrane fraction. The recombinant E. coli cells harboring the plasmid PET-A produced AmyA in the cytoplasmic fraction only, rather than in the membrane fraction (Figure 1), indicating that AmyA that lacked NCgl1221423 anchor can not be expressed in the cell membrane. On the other hand, the results suggested that NCgl1221423 anchor has the ability to attach the passengers to the cell membrane of E. coli.

Immunofluorescence microscopy and flow cytometric analysis

To further confirm the successful display of AmyA on the cell surface of E. coli by NCgl1221423 anchor, the immunofluorescence microscopy was performed. Immunofluorescent labeling of E. coli cells was carried out by mouse anti-FLAG M2 antibody and goat anti-mouse IgG (H + L) conjugated with Alexa Fluor 488. As shown in Figure 2, the green fluorescence of the immunostained NCgl1221423-AmyA fusion protein was observed in E. coli cells harboring the plasmid PET-NA; cells harboring the plasmids PET-28a and PET-A were not immunostained. This confirmed that AmyA was successfully attached and displayed on the surface of E. coli cells harboring the plasmid PET-NA by using NCgl1221423 as the anchoring protein. The result also indicated that NCgl1221423 contains a robust surface anchor to attach large proteins to the E. coli cell surface,
which was consistent with Western blot analysis. Flow cytometry was performed to quantitatively analyze the cell-surface-displayed enzyme. The cell surface-displayed AmyA-FLAG was stained with the first and second antibodies mentioned earlier, and E. coli cells harboring the plasmid PET-28a were used as a control for flow cytometry. As shown in Figure 3, cells harboring the plasmid PET-NA showed significantly higher fluorescence signal intensity than the cells harboring the plasmid PET-A. This result suggested that AmyA-FLAG was displayed on the cell surface of E. coli harboring the plasmid PET-NA, which was agreed with that obtained by immunofluorescence microscopy (Figure 2). All of these results indicated that NCgl1221_{423} could be used as a new anchor in the E. coli cell surface display system.

### Enzyme activity

There have been many research projects aimed at the development of E. coli cell surface display system for biotechnological and industrial applications such as whole-cell biocatalysis, biosorption, and live vaccines (Lee et al., 2004; Richins et al., 1997; Lee et al., 2003; Jose and von Schwichow, 2004). In order to determine whether AmyA expressed in the recombinant E. coli cells has the activity or not, we separated subcellular fractions and assayed for AmyA activity (Table 2). E. coli cells harboring PET-28a or PET-A or PET-NA were induced by IPTG for 8 h to determine AmyA activity. As expected, AmyA activity was observed both in the cytoplasmic and outer membrane fractions derived from E. coli cells.
hosting PET-NA (Table 2). The activity of AmyA in the outer membrane reached 46.3 ± 1.5 U/l after 8 h of induction, which was approximately a half of that in the cytoplasm (Table 2). The result suggested that AmyA in an active form was successfully attached to the cell membrane by NCgl1221_423, though most of the NCgl1221_423-AmyA fusion protein was expressed in the cytoplasm. The possible reason for the low level of AmyA activity in the outer membrane could be explained by the low anchor efficiency of the NCgl1221_423-AmyA fusion protein due to its large size. Therefore, most of the large fusion protein was detained in the cytoplasm. The activity of AmyA that lacked NCgl1221_423 anchor was detected only in the cytoplasmic fractions from E. coli hosting PET-A and reached 128.4 ± 2.6 U/l after 8 h of induction (Table 2). These results indicated that NCgl1221_423 from C. glutamicum indeed plays a role in anchoring the active enzyme toward the cellular membrane of E. coli. For further confirmation of active AmyA displayed on the cell surface, we collected recombinant E. coli cells and directly assayed for AmyA activity. AmyA activity on the cell surface of E. coli harboring PET-NA reached 48.7 ± 1.6 U/l after 8 h of induction, while no activity was detected in intact cells of E. coli harboring PET-28a or PET-A (Table 2). AmyA activity on E. coli cell surface reached the maximum value of 88.3±2.4 U/l at 16 h of induction and maintained stationary phase after 16 to 20 h of cultivation (data not shown). NCgl1221_423 was successfully developed as the anchoring protein to display the large protein with an active form on E. coli surface. This is the first report of the application of NCgl1221_423 to the E. coli cell surface display system. This novel system is applicable to wide fields, such as microbial biocatalysts, biosensors and protein libraries.

**Conclusion**

In summary, we developed a novel E. coli cell surface display system by using NCgl1221_423 as the anchoring protein. The α-amylase (AmyA) from S. bovis 148 was used as a model protein for display on the cell surface. We fused AmyA to the C terminus of the NCgl1221_423 anchor protein to display AmyA on the E. coli surface.
Figure 3. Flow cytometric analysis of *E. coli* harboring PET-28a, PET-A and PET-NA. Cells were labeled with mouse anti-FLAG M2 antibody, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG. Shaded area cells harboring the empty plasmid PET-28a. Unshaded area cells harboring PET-A for expression of AmyA, and cells harboring PET-NA for expression of the NCgl1221_{423}-AmyA fusion protein.

Table 2. The activity of α-amylase in different *E. coli* cells after 8 h of IPTG induction.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>α-Amylase activity (U/l)^a</th>
<th>Cytoplasm</th>
<th>Outer membrane</th>
<th>Intact cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET-28a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PET-A</td>
<td>128.4 ± 2.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PET-NA</td>
<td>102.1 ± 2.2</td>
<td>46.3 ± 1.5</td>
<td>48.7 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

^a The data represent the mean value and standard deviation of three independent experiments ND, not detected.

The results showed that the fusion protein was successfully expressed and displayed on the cell surface of *E. coli*. It was suggested that the NCgl1221_{423} protein can be utilized as an anchor protein in *E. coli* and the transmembrane structure of NCgl1221_{423} is not affected by its C-terminus fusion. The AmyA activity detected in subcellular fractions and in intact cells indicated that active AmyA was displayed on the *E. coli* cell surface.

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