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Display of Proteins on the Surface of *Escherichia coli* by C-Terminal Deletion Fusion to the *Salmonella typhimurium* OmpC

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Abstract A new system for displaying proteins on the surface of *Escherichia coli* was developed using the *Salmonella typhimurium* outer membrane protein C (OmpC) as an anchoring motif. The C-terminal deletion-fusion strategy was developed to fuse the polyhistidine peptides and green fluorescent protein (GFP) to the C-terminal of the truncated functional portion of OmpC. The polyhistidine peptides of up to 243 amino acids could be successfully displayed on the *E. coli* cell surface, which allowed recombinant *E. coli* to adsorb up to 34.2 μmol of Cd^{2+} per gram dry cell weight. The GFP could also be successfully displayed on the *E. coli* cell surface. These results suggest that the C-terminal deletion-fusion strategy employing the *S. typhimurium* OmpC as an anchoring motif provides a new efficient way for the display of large proteins on the surface of *E. coli*.

Key words: Cell surface display, *Salmonella* OmpC, anchoring motif, polyhistidine, GFP

Cell surface display (CSD) 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 [4, 7, 9, 12, 19]. The surface protein to be used as an anchoring motif should possess an efficient signal sequence for facilitating the translocation of a foreign protein through the inner membrane of the cell, a targeting signal for anchoring foreign protein to the surface of the cell in a stable manner, and a capability of accommodating foreign proteins or peptides of various sizes. Furthermore, it would be beneficial if the fusion protein can be expressed in large amounts [7, 12, 19]. CSD can be employed for a wide range of biotechnological and industrial applications, such as live vaccine development [11], peptide libraries screening [2, 13], whole cell catalysis [8], biosensor development [6, 17], and environmental bioadsorption [18, 20].

We have recently reported successful sandwich fusion display of polyhistidine of varying sizes on the surface of *Escherichia coli* by using the *E. coli* outer membrane protein C (OmpC) as an anchoring motif [20, 21]. Even though poly-(6His) peptide of up to 162 amino acids could be displayed using this sandwich fusion system, attempts to display larger polypeptide or enzyme were not successful. Difficulty in displaying a large protein has been a common problem in microbial cell surface display [12]. Therefore, we decided to explore and develop an alternative cell surface display system which allows expression and display of foreign polypeptides/proteins of larger sizes. In this paper, we report the development of a new cell surface displaying system based on the *Salmonella typhimurium* OmpC employing the C-terminal deletion-fusion strategy, which allowed the display of larger poly-(6His) peptide and functional green fluorescent protein (GFPuv).

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are summarized in Table 1. *E. coli* XL1-Blue and BL21(DE3) were used as host strains for the display of poly-(6His) peptides and GFP. For flask cultures, Luria-Bertani (LB) medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) was used. Cells were cultivated in 250-ml flasks containing 50 ml LB medium supplemented with 30 μg/ml kanamycin

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

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
XL1-Blue	*SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F'(proAB⁺ lacI^q lacZΔM15 Tn10(tet^r))*	Stratagene[a]
BL21(DE3)	F⁻ *ompT hsdS$_B$(r_B^- m_B^-) gal dcm (DE3)*	Novagen[b]
Salmonella typhimurium		KCTC 2053
Plasmids		
pUC19	Ap^r	New England Biolabs[c]
pACYC177	Ap^r, Km^r	New England Biolabs[c]
pGFPuv	Ap^r	Clontech[d]
pET3a	Ap^r, T7 promoter	Novagen[b]
pT7SC	Ap^r, OmpC, T7 promoter	This study
pT7KSC	Km^r, OmpC, T7 promoter	This study
pT7KSC-Hn	Km^r, OmpC, T7 promoter, n sets of polyhistidine linker	This study
pT7KSC-GFP	Km^r, OmpC, T7 promoter, GFPuv	This study

[a]Stratagene Cloning Systems, La Jolla, CA, U.S.A.
[b]Novagen, Inc., Madison, WI, U.S.A.
[c]New England Biolabs, Beverly, MA, U.S.A.
[d]Clontech Lab. Inc., Palo Alto, CA, U.S.A.

(Km) in a shaking incubator at 30°C and 200 rpm. At the cell density (OD_{600}) of 0.6, IPTG was added to a final concentration of 0.01 (for poly(6His)) or 0.1 mM (for GFPvu), followed by further cultivation for 2 h. Dry cell weight (DCW) was determined as described previously [3].

Plasmids and DNA Manipulation

The *S. typhimurium ompC* gene was cloned from *S. typhimurium* KCTC2053 (Korea Collection for Type Cultures, Daejeon, Korea) by PCR using the following primers: 5'-CTGCGCCTGGTCTCATATGAAAGTTAAAGTACTG-3' containing the *Nde*I site (underlined), and 5'-CCGGGATCC-TTATTAGAACTGGTAAACCAG-3' containing the *Bam*HI site (underlined). To construct a surface expression vector, the entire *S. typhimurium ompC* gene, including the signal sequence, was inserted into the *Nde*I-*Bam*HI site of pET3a to make pT7SC (Fig. 1). The Km^r gene from pACYC177 was inserted into the *Dra*I-*Eco*RI site of pT7SC to make pT7KSC (Fig. 1). Expression vectors that contain varying copies of poly-(6His) linkers were constructed (Figs. 1 and 2A). The poly-(6His) linker was obtained by overlapping PCR using the following primer pairs: 5'-GATAGATATC-CTGCAGGTCGACCCAAGCGGACATCACCATCATC-ACCAT-3' containing the *Pst*I and *Sal*I sites (underlined) and 5'-CCAAGGATCCGATATCCTCGAGACCAGAAT-GGTGATGATGGTGATG-3' containing the *Bam*HI and *Xho*I sites (underlined). The purified DNA fragment contains the poly-(6His) linker which encodes DPSGHHHHHHSGL peptide. This DNA fragment was cloned into the *Pst*I-*Bam*HI-digested pT7KSC to make pT7KSC-H1 (Fig. 2A). In order to construct plasmid pT7KSC-H2 that contains 2 sets of poly-(6His) linker, the PCR product digested with *Sal*I and *Xho*I was inserted into the *Sal*I site of pT7KSC-H1. Plasmids pT7KSC-H3, pT7KSC-H6, pT7KSC-H12, and pT7KSC-H18 were constructed in a similar manner as above (Fig. 2A), and they contained 3, 6, 12, and 18 sets of poly-(6His) linkers, respectively (Fig. 2A). The GFPuv gene [5] was obtained by PCR using the following primer pairs from the plasmid pGFPuv (Clontech lab. Inc, Palo Alto, CA, U.S.A.): 5'-AACTGCAGAGTAAAGGAGAAG-AACTTTTC-3' containing the *Pst*I site (underlined) and 5'-CGGGATCCTTATTTGTAGAGCTCATCCAT-3' containing the *Bam*HI site (underlined). The PCR product was digested with *Pst*I and *Bam*HI, and was cloned into the same sites of pT7KSC to make pT7KSC-GFP (Fig. 2A). PCR was performed with a PCR Thermal Cycler MP TP3000 (Takara Shuzo Co., Shiga, Japan) using High Fidelity PCR System (Boeheinger Mannheim, Mannheim, Germany). All DNA manipulations were carried out according to the procedures described in Sambrook *et al.* [16]. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.) and were used as recommended by the manufacturer.

Analytical Procedures

Outer membrane proteins were prepared and analyzed as described by Xu and Lee [20]. Protein samples were analyzed by 12% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [10]. Fractionated protein samples were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Recombinant *E. coli* expressing OmpC-$(6His)_n$ fusion proteins were examined for their abilities to adsorb Cd^{2+}. Induced cells were washed twice with 0.85% (w/v) NaCl, and were resuspended in 0.85% (w/v) NaCl (pH 5.8) to

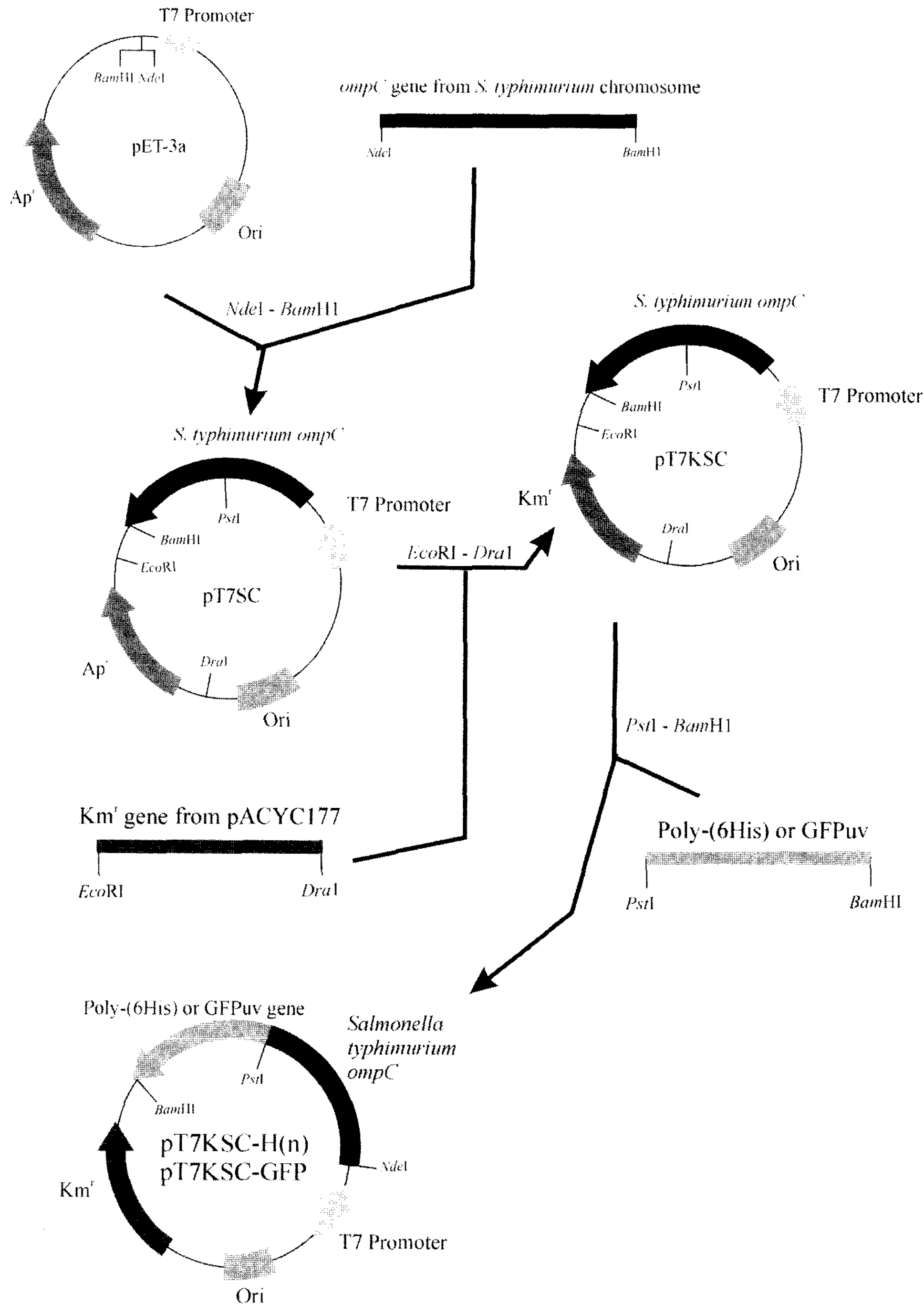

Fig. 1. Construction of plasmids used in this study.

give a cell concentration of 10 g DCW/L. An equal volume of $CdCl_2$ [50 ppm in 0.85% (w/v) NaCl, pH 5.8] was added, and the mixture was incubated for 24 h at 25°C with shaking. Cells were pelleted, washed twice with 0.85% (w/v) NaCl, and then were digested overnight with 70% (w/v) nitric acid at room temperature. Samples were analyzed by Atomic Analysis System (model 3300, Perkin-Elmer, Shelton, CT) using an air-acetylene flame and a hollow cathode lamp. The wavelength and slit width were 228.8 nm and 0.7 nm, respectively [20].

For the Western blot analysis, the protein bands on the gels were electrotransferred onto polyvinylidene-fluoride (PVDF) membrane (Sigma, St. Louis, MO, U.S.A.). The blotted membrane was placed in a blocking solution containing 5% (w/v) non-fat skim milk in PBST buffer [80 mM Na_2HPO_4, 20 mM NaH_2HPO_4, 100 mM NaCl, and 0.1% (v/v)

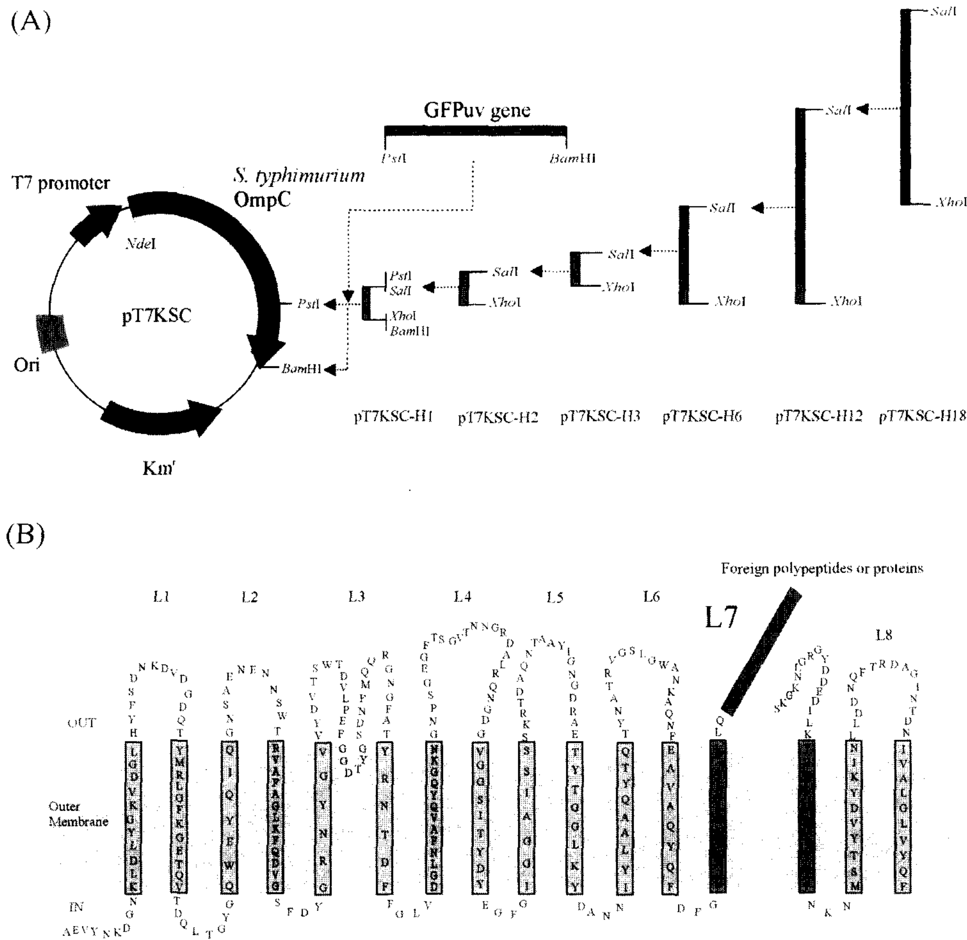

Fig. 2. (A) Construction of the plasmids pT7KSC-Hn and pT7KSC-GFP. (B) The topology of *S. typhimurium* outer membrane protein C on the outer membrane.
Poly-(6His) or GFPuv was fused at the L7 by using the *Pst*I site present within.

Tween-20; pH 7.5] for 1 h. For the immunodetection, primary rabbit polyclonal antibody against GFPuv (Molecular Probes Inc, Eugene, OR, U.S.A.) diluted 1:2,000 in PBST buffer was used. The secondary antibody used was horseradish peroxidase conjugated goat anti-rabbit IgG (HRP; Amersham Biosciences, Uppsala, Sweden) diluted 1:1,000 in PBST buffer.

Results and Discussion

Development of a Surface Display Vector Based on the C-Terminal Deletion-Fusion Strategy

In order to develop a method for displaying larger polypeptides or proteins on the cell surface of *E. coli* using the *S. typhimurium* OmpC as an anchoring motif, the C-terminal deletion-fusion strategy was developed. The OmpC is a major outer membrane protein, the expression of which is influenced by various environmental factors such as pH, osmolarity, and temperature [15]. It consists of 16 membrane-spanning β-strands which are antiparallelly aligned to form a β-barrel structure surrounding relatively nonspecific channels to allow the rapid passage of small hydrophilic molecules across the outer membrane (Fig. 2B). It was shown from the previous study by Xu and Lee [20] that the L7 loop of the *E. coli* OmpC is able to accept peptides up to 162 amino acids, which are larger than other sandwich fusion systems based on *E. coli* PhoE [1] and FimH [14]. We, therefore, chose this L7 site of the *Salmonella* OmpC as the C-terminal deletion-fusion point for possibly displaying larger polypeptides/proteins (Fig. 2B).

To construct a surface display vector, the entire *S. typhimurium ompC* gene, including the signal sequence, was inserted into the *Nde*I-*Bam*HI site of pET3a to make pT7SC. The Kmʳ gene from pACYC177 was cloned into the *Dra*I-*Eco*RI site of pT7SC, thus replacing the Apʳ gene, to construct pT7KSC. In this way, the *Pst*I site present in the Apʳ gene, which makes it inconvenient to clone a gene of interest into the *Pst*I site of the *ompC* gene,

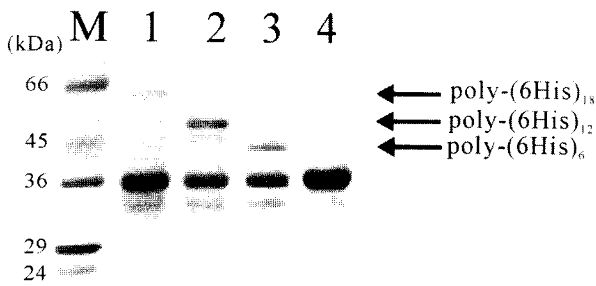

Fig. 3. SDS-PAGE analysis of outer membrane proteins from *E. coli* BL21(DE3) harboring pT7KSC-H6, pT7KSC-H12, and pT7KSC-H18.

Cells were cultivated at 30°C and were grown for an additional 2 h after induction with 0.01 mM of IPTG. Lanes are: M, molecular weight marker; 1, *E. coli* BL21(DE3) (pT7KSC-H18); 2, *E. coli* BL21(DE3) (pT7KSC-H12); 3, *E. coli* BL21(DE3) (pT7KSC-H6); 4, *E. coli* BL21(DE3). The arrows indicate OmpC-poly-(6His) fusion protein bands.

was deleted. To examine the ability to display foreign peptides and proteins, the poly-(6His) linkers of varying sizes and the GFPuv gene were cloned into the *Pst*I-*Bam*HI site of pT7KSC (Fig. 1). By doing so, the C-terminal 67 amino acids of the OmpC were deleted, and were replaced by the foreign polypeptide/protein to be displayed (Fig. 2B).

Display of Poly-(6His) Peptides and Green Fluorescent Protein (GFPuv) on the *Escherichia coli* Cell Surface

First, poly-(6His) linkers of varying lengths were used as model polypeptides to be displayed. The PCR-amplified DNA fragments coding for poly-(6His) linkers were inserted into the *Pst*I-*Bam*HI digested pT7KSC to construct pT7KSC-Hn (Fig. 2A). The localization of target polypeptides on the external surface of *E. coli* BL21(DE3) harboring pT7KSC-Hn was determined by metal ion adsorption and SDS-PAGE analysis. Figure 3 shows the SDS-PAGE of outer membrane proteins from recombinant *E. coli* BL21(DE3) harboring pT7KSC-H6, pT7KSC-H12, and pT7KSC-H18. The expression level of OmpC-$(6His)_{18}$ was 13.7% of the total outer membrane proteins. Xu and Lee [20] previously reported that the *E. coli* OmpC-$(6His)_{18}$ could not be displayed using sandwich fusion method. Therefore, it seems that the C-terminal deletion-fusion method is superior to the sandwich fusion method for the display of larger polypeptide, using OmpC as an anchoring motif.

Recombinant *E. coli* cells displaying OmpC-$(6His)_n$ fusion proteins were examined for their abilities to adsorb Cd^{2+}. Cells harboring pT7KSC-H6, pT7KSC-H12, and pT7KSC-H18 could adsorb 23.9, 30.8, and 34.2 µmol of Cd^{2+} per gram DCW, respectively, while the control strain harboring pT7KSC adsorbed 11.1 µmol of Cd^{2+} per gram DCW. The highest Cd^{2+} removal capacity achieved using pT7KSC-H18 was higher that that achieved using the OmpC sandwich fusion display system by Xu and Lee [20].

Next, GFPuv was used as a model protein to be displayed on the *E. coli* cell surface. GFPuv is a globular protein (27 kDa) and is a versatile reporter protein in biological research for protein localization studies, monitoring gene expression and detecting bacteria in the environment. Samples of outer membrane fraction were prepared from the induced cultures of *E. coli* BL21(DE3) harboring pT7KSC-GFP, and were analyzed by SDS-PAGE (Fig. 4). The SDS-PAGE analysis showed that the total and outer membrane fractions contained the OmpC-GFPuv fusion protein bands. As shown in Fig. 4, the bands of expected size (56 kDa) in the outer membrane and total fraction were recognized by anti-GFP polyclonal antibody, suggesting that *E. coli* BL21(DE3) cells harboring pT7KSC-GFP indeed expressed GFPuv on the cell surface. When illuminated by UV lights, these cells harboring pT7KSC-GFP were fluorescent. These results confirm that the C-terminal deletion-fusion strategy allowed successful display of GFPuv using the truncated OmpC as an anchoring motif. It should be mentioned that GFPuv could not be displayed, when the OmpC sandwich fusion method was employed using the OmpC as an anchoring motif (data not shown).

Several cell surface display systems have been developed for the expression of polypeptides or proteins on the surface of *E. coli*. Most of the surface display systems using outer membrane protein as an anchoring motif are

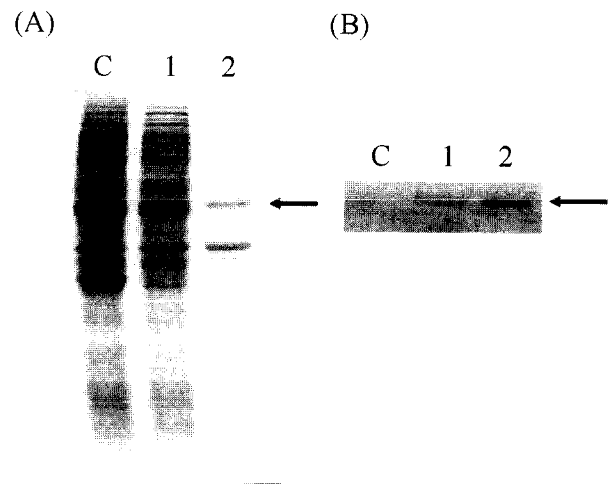

Fig. 4. (A) SDS-PAGE analysis of OmpC-GFPuv fusion protein displayed on the surface of *E. coli.*

Cells were cultivated at 30°C and were grown for an additional 5 h after induction with 0.1 mM IPTG. Lanes are: C, total proteins from *E. coli* BL21(DE3) harboring pGFPuv; 1, total proteins from *E. coli* BL21(DE3) harboring pT7KSC-GFP; 2, outer membrane proteins from *E. coli* BL21(DE3) harboring pT7KSC-GFP. The arrow indicates OmpC-GFPuv fusion protein. (B) Western blot analysis of OmpC-GFPuv fusion proteins using rabbit polyclonal antibody against GFPuv. Lanes are: C, total proteins from *E. coli* BL21(DE3) harboring pGFPuv; 1, total proteins from *E. coli* BL21(DE3) harboring pT7KSC-GFP; lane 2, outer membrane proteins from *E. coli* BL21(DE3) harboring pT7KSC-GFP. The arrow indicates OmpC-GFPuv fusion protein.

based on a sandwich fusion method. The most widely used systems include the maltoporin LamB, the outer membrane protein S, and the lipoprotein TraT [12]. However, the passengers displayed have been rather limited to short epitopes or polypeptides. In this study, we used a C-terminal deletion fusion strategy using the *Salmonella* OmpC as an anchoring motif, which allowed successful display of larger poly-(6His) polypeptide and GFPuv. These results suggest that the C-terminal deletion-fusion strategy employing the *S. typhimurium* OmpC is suitable for the display of large polypeptides and proteins, which should prove to be useful in a wide range of applications.

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REFERENCES

1. Agterberg, M., H. Adriaanse, A. van Bruggen, M. Karperien, and J. Tommassen. 1990. Outer-membrane PhoE protein of *Escherichia coli* K-12 as an exposure vector: Possibilities and limitations. *Gene* **88:** 37-45.
2. Boder, E. T. and K. D. Wittrup. 1997. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15:** 553-557.
3. Choi, J., S. Y. Lee, K. Shin, W. G. Lee, S. J. Park, H. N. Chang, and Y. K. Chang. 2002. Pilot scale production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant *Escherichia coli. Biotechnol. Bioproc. Eng.* **7:** 1-4.
4. Cornelis, P. 2000. Expressing gene in different *Escherichia coli* compartments. *Curr. Opin. Biotechnol.* **11:** 450-454.
5. Crameri, A., E. A. Whitehorn, E. Tate, and W. P. C. Stemmer. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* **14:** 315-319.
6. Dhillon, J. K., P. D. Drew, and A. J. Porter. 1999. Bacterial surface display of an anti-pollutant antibody fragment. *Lett. Appl. Microbiol.* **28:** 350-354.
7. Georgiou, G., C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson, and R. Curtiss III. 1997. Display of heterologous proteins on the surface of microorganisms: From the screening of combinatorial libraries to live recombinant vaccines. *Nat. Biotechnol.* **15:** 29-34.
8. Jung, H. C., J. M. Lebeault, and J. G. Pan. 1998. Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae. Nat. Biotechnol.* **16:** 576-580.
9. Kim, K. Y., M. D. Kim, N. S. Han, and J. H. Seo. 2002. Display of *Bacillus macerans* cyclodextrin glucanotransferase on cell surface of *Saccharomyces cerevisiae. J. Microbiol. Biotechnol.* **12:** 411-416.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
11. Lee, J. S., K. S. Shin, J. G. Pan, and C. J. Kim. 2000. Surface-displayed viral antigens on *Salmonella* carrier vaccine. *Nat. Biotechnol.* **18:** 645-648.
12. Lee, S. Y., J. H. Choi, and Z. Xu. 2003. Microbial cell-surface display. *Trends Biotechnol.* **21:** 45-52.
13. Martineau, P., A. Charbit, C. Leclerc, C. Werts, D. O'Callaghan, and M. Hofnung. 1991. A genetic system to elicit and monitor anti-peptide antibodies without peptide synthesis. *Bio/Technology* **9:** 170-172.
14. Pallesen, L., L. K. Poulsen, G. Christiansen, and P. Klemm. 1995. Chimeric FimH adhesin of type 1 fimbriae: A bacterial surface display system for heterologous sequences. *Microbiology* **141:** 2839-2848.
15. Pratte, L. A., W. Hsing, K. E. Gibson, and T. J. Silhavy. 1996. From acids to osmZ: Multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli. Mol. Microbiol.* **20:** 911-917.
16. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd Ed. Cold Spring Harbor Laboratory Press, NY, U.S.A.
17. Shibasaki, S., M. Ueda, K. Ye, K. Shimizu, N. Kamasawa, M. Osumi, and A. Tanaka. 2001. Creation of cell surface-engineered yeast that display different fluorescent proteins in response to the glucose concentration. *Appl. Microbiol. Biotechnol.* **57:** 528-533.
18. Sousa, C., P. Kotrba, T. Ruml, A. Cebolla, and V. de Lorenzo. 1998. Metalloadsorption by *Escherichia coli* cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB. *J. Bacteriol.* **180:** 2280-2284.
19. Stahl, S. and M. Uhlen. 1997. Bacterial surface display: Trends and progress. *TIBTECH* **15:** 185-192.
20. Xu, Z. and S. Y. Lee. 1999. Display of polyhistidine peptides on the *Escherichia coli* cell surface by using outer membrane protein C as an anchoring motif. *Appl. Environ. Microbiol.* **65:** 5142-5147.
21. Xu, Z., S. Y. Lee, and Z. Yu. 1999. Physiological characteristics of recombinant *Escherichia coli* displaying poly-His peptides. *Biotechnol. Lett.* **21:** 1091-1094.