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Surface display of human arginase1 through engineered antigen 43 in *Escherichia coli*

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ABSTRACT

***Escherichia coli* auto-transporter protein antigen 43 (Ag43) was one of the superfamily of auto-transporters (AT) which was considered to be a potentially useful anchor for bacteria surface display because of its relative simplicity and high copy numbers. Here, the system used four charged polypeptides [6×Lys, 6×Glu and 6×Asp and 2×(Lys/Lys/Arg)] was engineered to replace its native signal peptide for the surface display of human Arginase1 (ARG1) in *E. coli*. The results obtained indicate that charged polypeptides 6×Lys, 6×Glu and 2×(Lys/Lys/Arg) could significantly enhance the display efficiency of human ARG1. Among these tags, the 6×Lys polypeptide had the highest display efficiency (37.73%) which was about 7.6-fold of the original Ag43 system (4.91%). The whole cell enzyme activity test also confirmed that optimized system (K6-ARG1-Ag43) obtained the highest activity of 15.21 U/mL(OD_{600}=1.0), which was about 22-fold of the original Ag 43 system (0.69 U/mL, OD_{600}=1.0). Optimized system (K6-ARG1-Ag43) also possessed high stability, after 10 repeated reaction cycles, the whole cell enzyme activity remained more than 35% of its initial activity. Furthermore, almost 100% of L-arginine (200 g/L) transferred into L-ornithine (L-Orn) in 16 h under the optimum condition in the batch conversion test. The engineered Ag43 display system expanded the Ag43 surface display system and provided alternative methods for display of human Arginase1 in *E. coli*.**

INTRODUCTION

Bacterial cell surface display describes the exposure of recombinant proteins or peptides on cell surface through different carrier proteins or anchor proteins; and this has obtained a major interest in biotechnology and medicine (Lee et al., 2003; Rutherford and Mourez, 2006). Display of enzymes on bacteria surface was used as whole-cell biocatalysts for bioconversion, especially in industrial applications. Compared with purified enzymes method, whole-cell bioconversion was a promising way due to its high efficiency, relatively easy preparation prior to their use and low cost (Schüürmann et al., 2014; Jose et al., 2012). Many surface appendage proteins have been used as carrier or anchor proteins for displaying of heterologous proteins, such as ice nucleation protein, S-layer protein, OmpC, AIDA-I et al (Kjaergaard et al., 2002; Bielen et al., 2014; Bloois et al., 2011). Type V secretion system protein Antigen 43 (Ag43) contained information for translocation across the membrane and secretion in itself. Full length of Ag43 consisted of two domains, α and β domain, the β-domain formed a pore-like β-barrel structure inserted into the outer membrane after the α-domain was recognized by its signal peptide through Sec-pathway (Tozakidis et al., 2015; Kjaergaard et al., 2002). Heterologous proteins had been used to

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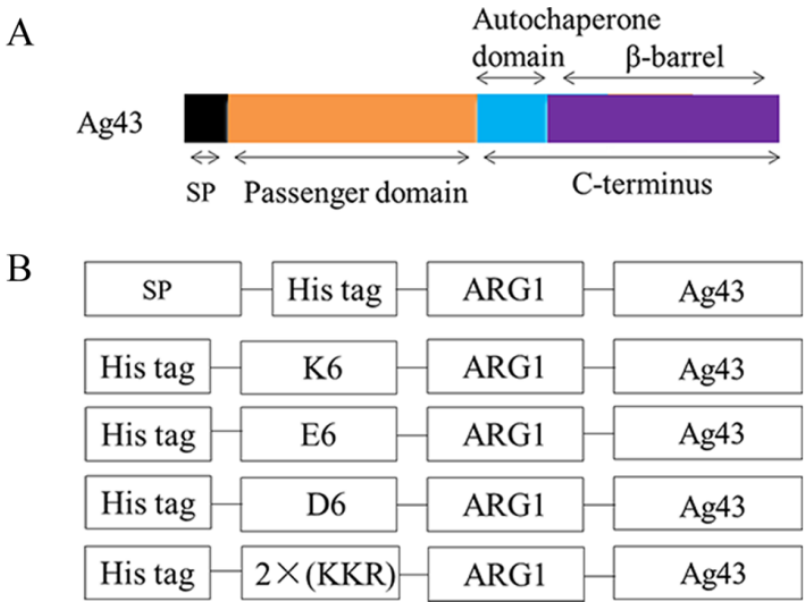

Figure 1. Constructs in the study. **(A)** Structure of Ag43 used in the study. SP, Signal peptide of native Ag43; C-terminus, auto-chaperone domain and β-barrel of Ag43, also termed as β-domain, or Ag43 in this study. Drawing is not to scale. **(B)** Different engineered Antigen 43 Display System used in the study.

replace the native passenger protein of Ag43 to anchor on the cell surface. Even more, the β-domain alone with its α-helix was sufficient to anchor recombinant proteins on the surface of *E. coli* (Ramesh et al., 2012).

Human Arginase1 (ARG1) converts L-arginine (L-Arg) to L-ornithine (L-Orn) and urea to discharge the redundant ammonia, which plays an important role in nitrogen metabolism. Diseases such as: spastic diplopia, intellectual disability, seizures and growth deficiency caused by the deficiency of ARG1 (Li et al., 2016). The bioconversion product L-Orn was a no-protein amino acid with various functions and applications, including the treatment of liver diseases, strengthen the heart function (Zhang et al., 2015). Meanwhile, arginase is a significant drug candidate for certain types of cancers which are auxotrophic for arginine in the near future (Rose et al., 1998). However, because human ARG1 has to form a trimer to perform its function, it was difficult to actively immobilize the enzyme on the cell surface, which limited its further engineering using surface display-mediated high-throughput methods.

In our previous work, human ARG1 had been successfully immobilized on the surface of *E. coli* through INP display system (Zhang et al., 2016). However, the surface display efficiency was relatively low as well as lower whole cell activity (the highest display efficiency was 14.8% and whole cell activity was 13.47 U/mL). In this report, optimization of a bacterial Ag43 surface display system was researched. C-terminus of Ag43 (contain β-barrel and auto-chaperone domain) from *E. coli* k-12 genome was amplified and used as the anchor protein for the display of human Arginase1 in *E. coli* (Figure 1A). For the optimization of this display system, charged polypeptides (Table 1) were used to replace the native signal peptide of Ag43 system (Figure 1B). The results obtained indicate that original Ag43 system with a low ARG1 displayed efficiency of 4.91%±0.08%, while polypeptides [6×Lys, 2×(Lys/Lys/Arg) and 6×Glu] could be significantly increase by 7.68-fold, 2.68-fold and 1.65-fold (37.33%±0.28%, 13.16%±0.02%, 8.11%±0.08%) (Figure 2C-E) respectively, when compared with the original Ag43 system. Further investigations demonstrated that engineered strain harboring plasmid pET-23a-His6·tag/K6-ARG1-Ag43 obtained the highest enzyme activity of 15.21 U/mL (OD_{600}=1.0) under the optimum reaction condition (Figure 3B). Optimized system (K6-ARG1-Ag43) also had high stability in batch reaction test, after 10 repeated reaction cycles, 35% of its initial activity was retained under the optimum temperature 50°C and pH 10.0 (Figure 4E). To our knowledge, the work presented here was the first report that human ARG1 could be actively displayed on the *E. coli* surface through an Ag43 display system. This new Ag43 display system had higher surface display efficiency (2.5-fold) and whole cell activity (1.1-fold) compared with our previous work (Zhang et al., 2016). As ARG1 was

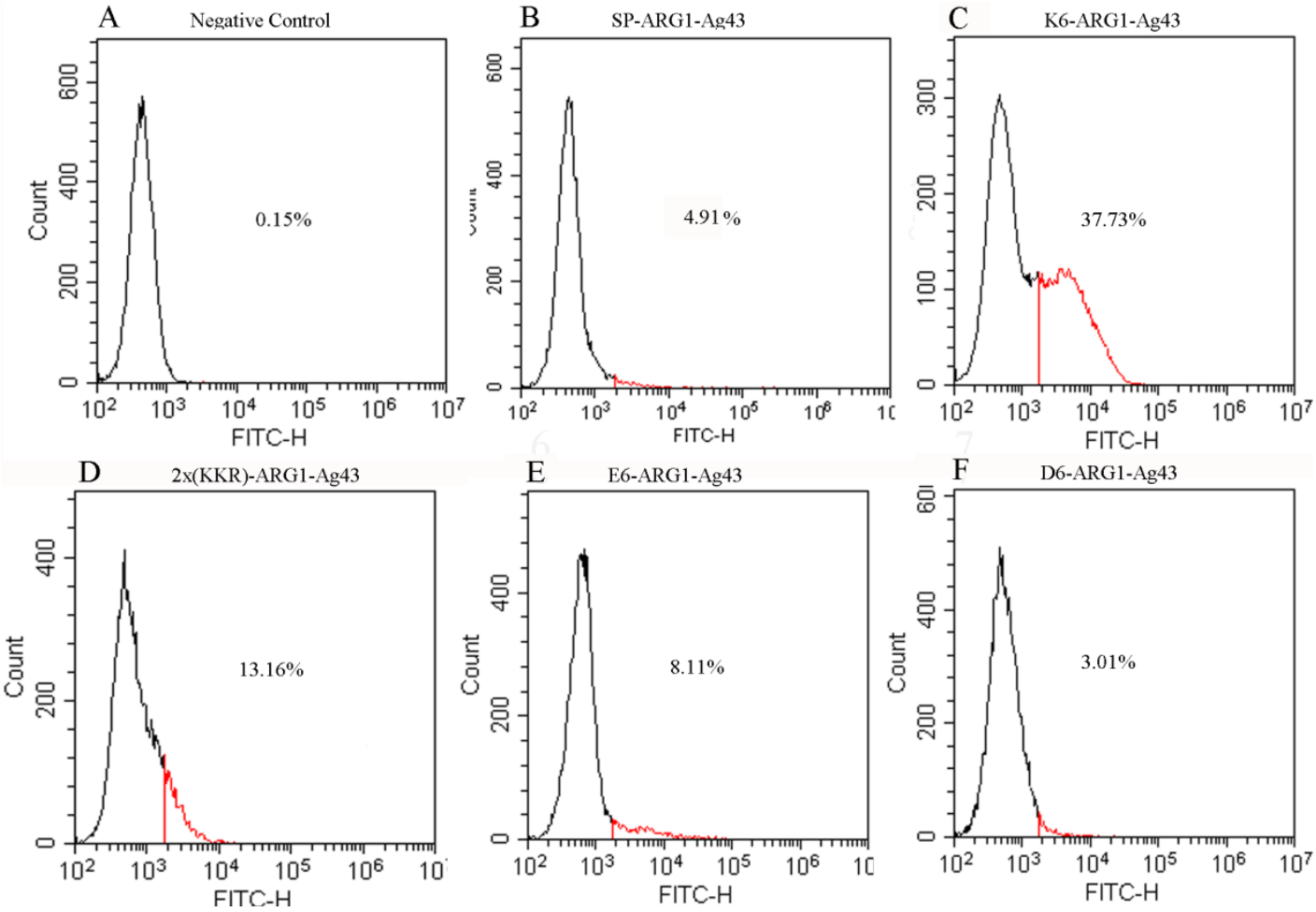

Figure 2. Flow cytometry assay of different engineered Ag43 display system. Recombinant strain contained different plasmids which are labeled: FITC – conjugated goat anti-mouse, IgG second antibody (see details in Materials and Methods), followed by the flow cytometric analysis. 1-6: cells harboring plasmid pET23a-T empty vector, SP-ARG1-Ag43, K6-ARG1-Ag43, 2×(KKR)-ARG1-Ag43, E6-ARG1-Ag43, D6-ARG1-Ag43.

Table 1. Amino acid sequences of signal and polypeptides used in this study.

Signal peptide/Polypeptide	Sequence[a]
Ag43 SP[b]	MKRHLNTCYRLVWNHMTGAFVVASELARARGKRGGVAVALSLAAVTSLPVLA
K6	KKKKKK
E6	EEEEEE
D6	DDDDDD
2×(KKR)	KKRKKR
Ag43[c]	ATLKVKNLNGQNGTISLRVRPDMAQNNADRLVIDGGRATGKTILNLVNAGNSASGLATSGKGIQVV EAINGATTEEGAFVQGNRLQAGAFNYSLNRDSDESWYLRSENAYRAEVPLYASMLTQAMDYDRIV AGSRSHQTGVNGENNSVRLSIQGGHLGHDNNGGIARGATPESSGSYGFVRLEGDLMRTEVAGM SVTAGVYGAAGHSSVDVKDDDGSRAGTVRDDAGSLGGYLNLVHTSSGLWADIVAQGTRHSMKAS SDNNDFRARGWGWLGSLETGLPFSITDNLMLEPQLQYTWQGLSLDDGKDNAGYVKFGHGSAQH VRAGFRLGSHNDMTFGEGTSSRAPLRDSAKHSVSELPVNWWVQPSVIRTFSSRGDMRVGTSTA GSGMTFSPSQNGTSLDLQAGLEARVRENITLGVQAGYAHSVSGSSAEGYNGQATLNVTF

The sequence is given in the N-to C-terminal direction; Ag43 SP[b] represents the native signal peptide of Ag43; Ag43[c] represents the C-terminus of Ag43 also termed as β-domain in this study.

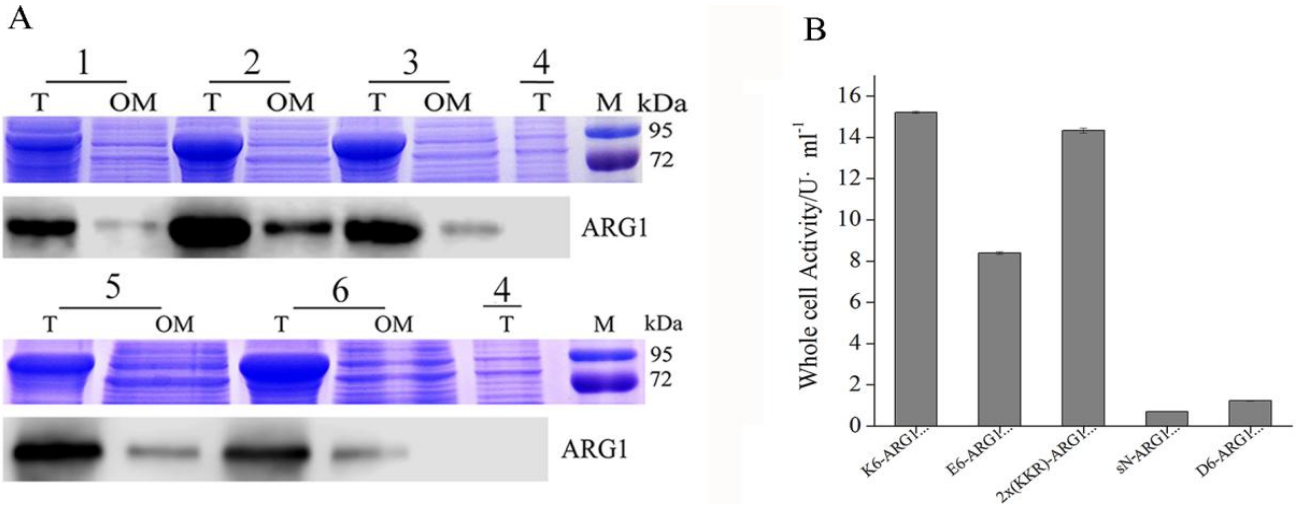

Figure 3. Surface display of human ARG1 on the *E. coli* cell outer membrane. **(A)** SDS-PAGE and Western-Blot analysis of human ARG1 on the outer membrane. Upper panel, the SDS-PAGE analysis, lower panel, western-blot analysis. M, protein ladder; T, total cell proteins; OM, outer membrane proteins. 1-6:SP-ARG1-Ag43,K6-ARG1-Ag43, 2×(KKR)-ARG1-Ag43, control with the empty pET23a-T-vector, D6-ARG1-Ag43,E6-ARG1-Ag43. **(B)** Whole cell enzyme activity. Each value represents the mean of three independent measurements and error bars correspond to the standard deviation of three independent determinations.

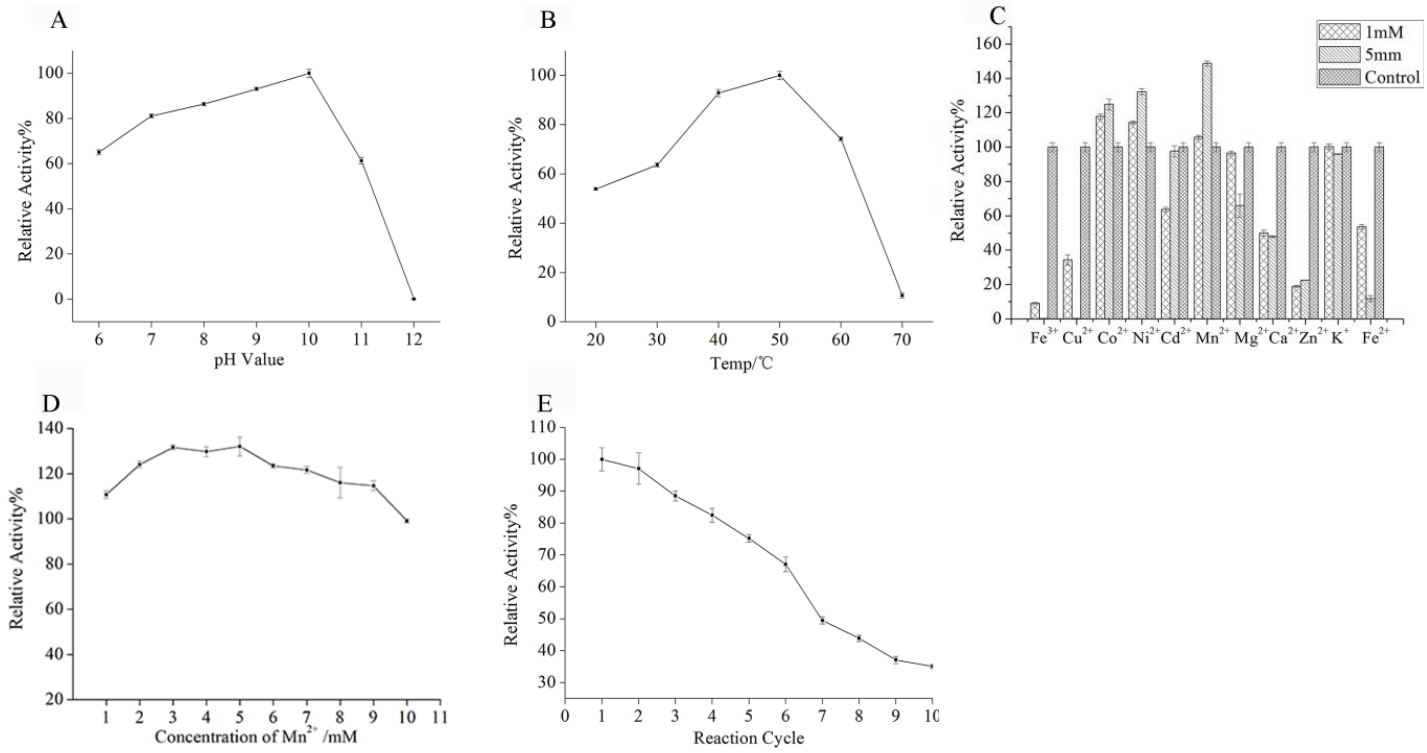

Figure 4. Characterization of the cell surface immobilized human ARG1. **(A)** Relative activity of strains harboring the recombinant plasmid K6-ARG1-Ag43 at different pH values. **(B)** Relative activity of strains harboring the recombinant plasmid K6-ARG1-Ag43 at different temperatures. **(C)** Influence of different metal ions on the cell's (strains harboring the recombinant plasmid K6-ARG1-Ag43) relative activity. **(D)** The influence of the concentration of Mn^{2+} in the reaction on the cell's relative activity (strains harboring the recombinant plasmid K6-ARG1-Ag43). **(E)** Operational stability of cells contained K6-ARG1/ag43 in the batch reaction cycles. The maximum activity was normalized as 100% in all the assays. Each value represents the mean of three independent measurements and error bars correspond to the standard deviation of three independent determinations.

Table 2. Bacterial strains and plasmids used in the study.

Strains and plasmids	Description	Sources
Strains		
E. coli XL10-Gold	Expression strain	Novagen (USA)
E. coli Rosetta Blue (DE3)	Cloning strain	Novagen (USA)
Plasmids		
pHBM905A-ARG Ⅰ	Gene ARG1	our laboratory
pET-23a-gfp	T-vector	our laboratory
pET-23a-His6·tag/ARG1-Ag43	original Ag43 system	This work
pET-23a-His6·tag/K6-ARG1-Ag43	K6-Ag43 system	This work
pET-23a-His6·tag/E6-ARG1-Ag43	E6-Ag43 system	This work
pET-23a-His6·tag/D6-ARG1-Ag43	D6-Ag43 system	This work
pET-23a-His6·tag/2×(KKR)- ARG1-Ag43	2×(KKR)-Ag43 system	This work

widely demanded in industry and cancer therapy (Li et al., 2016; Burrage et al., 2015), the findings from this study provide alternative methods for its application and engineering. Taken together, engineered Ag43 display system expanded the Ag43 surface display system, which provided a new choice for surface display and engineering of complex proteins in *E. coli*.

MATERIALS AND METHODS

Strains, plasmids and media

E. coli cells XL10-Gold and Rosetta Blue (DE3) were purchased from Novagen (USA). Human ARG1 coding region was synthesized and preserved by our own laboratory (Zhang et al., 2015). Prime STAR HS DNA Polymerase, Pfu DNA polymerase and Restriction endonuclease Bfu were purchased from TAKARA. Luria–Bertani (LB) medium was prepared for the cultivation of *E. coli* as described in the manualof molecular cloning. All chemicals used in this study were of analytical grade.All the antibodies were purchased from California Bioscience (USA). The strains and plasmids used in this study were listed in Table 2.

Vector construction and bacteria strains growth conditions

Genomic DNA of *E. coli* k-12 strain was used as template, primer pairs $F_{\beta}R_{\beta}$ and $F_{sp}R_{sp}$ were used to amplify gene of β-domain and native signal peptide of Ag43 (Figure 1A), respectively. Gene ARG1 was amplified from the plasmid constructed by our laboratory (Zhang et al., 2015) used the primers F_{arg1} and R_{arg1}. The above three gene fragments were used as templates, primer pairs $F_{sp}R_{\beta}$ were used to add the gene ARG1 between the signal peptide and the N-terminal of β-domain. Then the fused gene fragment was cloned into the pET-23a-T vector (Zhong et al., 2013) for expression interesting genes to construct recombinant plasmid pET-23a-His6·tag/ARG1-Ag43 (Figure 5A). Then the plasmid pET-23a-His6·tag/ARG1-Ag43 as template, four primer pairs (Table 3) were used to replace the native signal peptide of Ag43 (Figure 5B) to construct recombinant plasmids pET-23a-His6·tag/K6-ARG1-Ag43, pET-23a-His6·tag/E6-ARG1-Ag43, pET-23a-His6·tag/D6-ARG1-Ag43 and pET-23a-His6·tag/2×(KKR)-ARG1-Ag43. Primer pairs used in this study were listed in Table 3. The positive clones were identified by colony polymerase chain reaction (PCR) and confirmed by DNA sequence analysis.

All recombinant plasmids were transformed into the *E. coli* strain Rosetta Blue (DE3) for expression. Each single clone was picked and routinely grown at 37°C in 100 mL of LB medium with 50 μg/mL ampicillin until OD_{600} between 0.6 and 0.8, then cells were induced by IPTG as the final concentration of 1 mM by 18°C for another 12 h culturing at 200 rpm.

Outer membrane preparation and analysis

Outer membrane proteins were enriched according to the rapid isolation method (Richins et al., 1997). Samples were analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western-blot. For the SDS-PAGE analysis, samples were boiled with sample loading buffer (100 mMTris-Cl, pH 6.8, containing 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol, and 50 mg dithiothreitol) at 100°C for 10 min and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a 8% acrylamide resolving gel. After electrophoresis, gel was stained by coomassie Brilliant Blue. For western blot

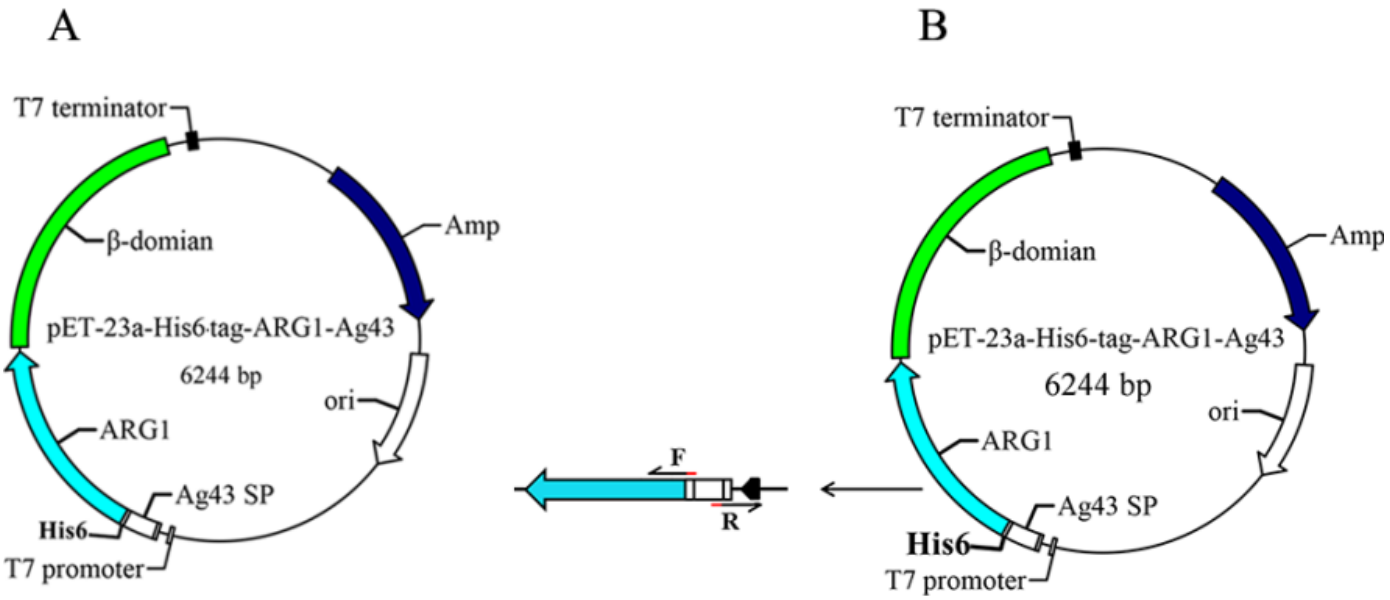

Figure 5. Schematic diagram of recombinant plasmid. **(A)** Schematic diagram of pET-23a-His6·tag/ARG1-Ag43. **(B)** Charged polypeptides were used to replace the native signal peptide pET-23a-His6·tag/ARG1-Ag43.

Table 3. Primers used in this study.

Primers	Oligonucleotide sequences (5'⟶ 3')
F_{-sp}	catcatcatcatcataaacgacatctgaatacctgctacaggctggta
R_{-sp}	atgatgatgatgatggtgagccagcaccgggagtgacgtgactg
F_{-arg1}	caccatcatcatcatcatggccttaacgatatctttgaggcccagaa
R_{-arg1}	accctgaaaatacaggttttcctttggtgggttcaaatagtcaattggt
$F_{-\beta}$	ctgtattttcagggtgcaaccctgaaagtgaaaaacctgaacg
$R_{-\beta}$	aattgtgagcgcttaagaaggtcacattcagtgtggcctga
F_{K6}	catcatcatcatcataaaaagaaaaagaaaaagggccttaacgatatc
R_{K6}	tttatgatgatgatgatggtgcatatgtatatctccttcttaaagttaaa
F_{E6}	catcatcatcatcatgaagaagaggaggaagagggcctta
R_{E6}	ttcatgatgatgatgatggtgcatatgtatatctccttcttaaagtt
F_{D6}	catcatcatcatcatgacgatgatgacgatgacggcct
R_{D6}	gtcatgatgatgatgatggtgcatatgtatatctccttctta
$F_{2\times(KKR6)}$	catcatcatcatcataaaaaacgcaaaaaacgcggccttaacgatatcttt
$R_{2\times(KKR6)}$	tttatgatgatgatgatggtgcatatgtatatctccttcttaaagtt

in H_2O, pH 7.6 and 0.05% Tween-20) with 5% skim milk for 1 h at 37°C. After washing with TBST buffer for three times, membranes were incubated with anti-his tag antibody (diluted 1:1000) at 37°C for 1 h. The membrane was washed three times with TBST buffer then further incubated with secondary antibody goat anti-mouse IgG conjugated with HRP (diluted 1:3000) at 37°C for another 1 h. Antigen-antibody conjugates were visualized by a color reaction with ECL Western Blotting Substrate (Thermo Pierce,USA).

Protein accessibility test

The protein accessibility test was performed to confirm the surface protein expression as previously described (Xiao-Jun et al., 2010). 200 µL cells were harvested and washed twice with phosphate-buffered saline (PBS) buffer, then centrifuged and re-suspended in proteinase K solution containing 15% (w/v) sucrose, 15 mMTris-HCl (pH 7.8), 0.1 mM EDTA and 200 mg of proteinase K. Cells were incubated at 37°C for 30 min, then whole cell

arginase activity was tested by Chinard reaction immediately (Chinard, 1952). One unit of the enzymatic activity was defined as the amount of enzymes needed to catalyze the release of 1 μM L-Orn per min at 50°C pH 10.0.

Fluorescence microscope and flow cytometry assay

For immunofluorescence studies, after washing three times with PBS buffer, 200 μL re-suspended cells (OD_{600} = 1.0) were blocked with PBS buffer containing 1% bovine serum albumin (BSA) at 4°C for 1 h. Cells were pelleted in a centrifuge and suspended in 200 μL PBS buffer, and labeled with anti-his tag antibody (diluted 1:1000) at 4°C for 1 h. Then, the cells were washed twice with 400 μL PBS buffer and re-suspended in 200 μL PBS buffer, followed by incubation with Fluorescein Isothiocyanate (FITC) – conjugated goat anti-mouse IgG second antibody in the dark at 4°C for another 1 h. Finally, the labeled cells were washed twice; and suspended in 200 μL PBS buffer. For the fluorescence microscopy observations, 7 μL of FITC – labeled samples were dipped onto a microscope slide and examined with a laser Zeiss LSM-710 confocal microscope (Jena, Germany) equipped with a 63× objective. Fluorophores were excited with an argon laser (488nm), using the EGFP detecting channel. For the flow cytometry assay, cells labeled with FITC were analyzed using the Cytoflexcell sorter (Beckman Coulter, USA).

Characteristics of engineered whole-cell immobilized ARG1

The final reaction mixture (1 mL) contained 20 mM of L-Arg (100 μL of 0.2 M L-Arg), enzyme source (100 μL cells, OD_{600}=1.0) of each samples and 800 μL of bicarbonate buffer (pH 10.0). After 10 min reaction, reaction mixture was centrifuged at 1,3000 g for 5 min immediately. The supernatant was transferred to new Eppendorf tube and heated at 100°C for 5 min to terminate the reaction. The L-Orn content in the reaction buffer was detected according to previous measurement (Zhang et al., 2015; Patchett et al., 1991). The catalyzed product L-Orn was also identified by Liquid chromatography - mass spectrometry (LC-MS) measurement.

To optimize the temperature, 100 μL cells (OD_{600}=1.0) were added in to 0.05 M bicarbonate buffer (pH 10.0), temperature ranging from 30-80°C. For optimal pH assay, cells were added in phosphate buffer (0.05 M, pH 6.0-7.0), Tris-HCl buffer (0.05 M, pH 7.0-8.0), bicarbonate buffer (0.05 M, pH 9.0-10.0) and hydrogen phosphate buffer (0.05 M, pH11.0-12.0) at 50°C. To optimize the metal ions, cells were added individually to 0.05 M bicarbonate buffer contains various metal ions at 50°C. All enzymes activities were assessed as described above, and each experiment was performed in triplicate independently.

RESULTS

Vector construction of engineered Ag43 display system

The native signal peptide and β-barrel domain of Ag43 was amplified from *E. coli* k-12 genomic DNA, which were 153 and 1332 bp, respectively. Gene ARG1 was amplified from recombinant plasmid by our lab, which was 1032 bp. Then, the above three fragments formed SP-ARG1-Ag43 (2496bp).

Conformation of engineered ARG1-ag43 anchored at the cell surface

To confirm the fact that recombinant human ARG1 was immobilized on the surface of *E. coli*, the outer membrane proteins containing human ARG1 fusion protein was fractionized. Then followed by analysis using SDS-PAGE and western-blot (Figure 3A). The bands were detected both in the total cell fractions and outer membrane extracts with the molecular weight as expected, including SP-ARG1-Ag43(84.9kDa), K6-ARG1-Ag43(85.7kDa), D6-ARG1-Ag43(85.6 kDa), E6-ARG1-Ag43 (85.7kDa) and 2×(KKR)-ARG1-Ag43 (85.7 kDa).

The engineered Ag43 systems for display foreign protein were further quantitated. As Human ARG1 formed a trimer structure, which was the main obstacle for its display on the cell membrane utilizing the previous surface display systems (Ramesh et al., 2012). In our study, human ARG1 was evaluated by engineered Ag43 surface display system. Different recombinant *E. coli* cells were labeled with FITC – conjugated fluorescent antibody and then assessed by fluorescence-activated cell sorting (FACS) analysis. Compared with original Ag43 system, cells harboring the plasmid pET-23a-His6·tag/D6-ARG1-Ag43 had relatively low fluorescent signals (Figure 2B and F), while cells harboring the plasmid pET-23a-His6·tag/E6-ARG1-Ag43, pET-23a-His6·tag/K6-ARG1-Ag43 and pET-23a-His6·tag/2×(KKR)-ARG1-Ag43 presented higher fluorescent signals, indicating that they could enhance the efficiency of surface display of human ARG1 (Figure 2C-E). Among all the engineered Ag43 systems, 37.73% ± 0.28% of the cells containing plasmid pET-23a-His6·tag/K6-ARG1-Ag43 presented fluorescent signals, which was almost 7.6-fold of original Ag43 system (4.91%±0.08%). Cells harboring plasmid pET-23a-His6·tag/2×(KKR)-ARG1-Ag43 and pET-23a-His6·tag/E6-ARG1-Ag43 could also increase the surface

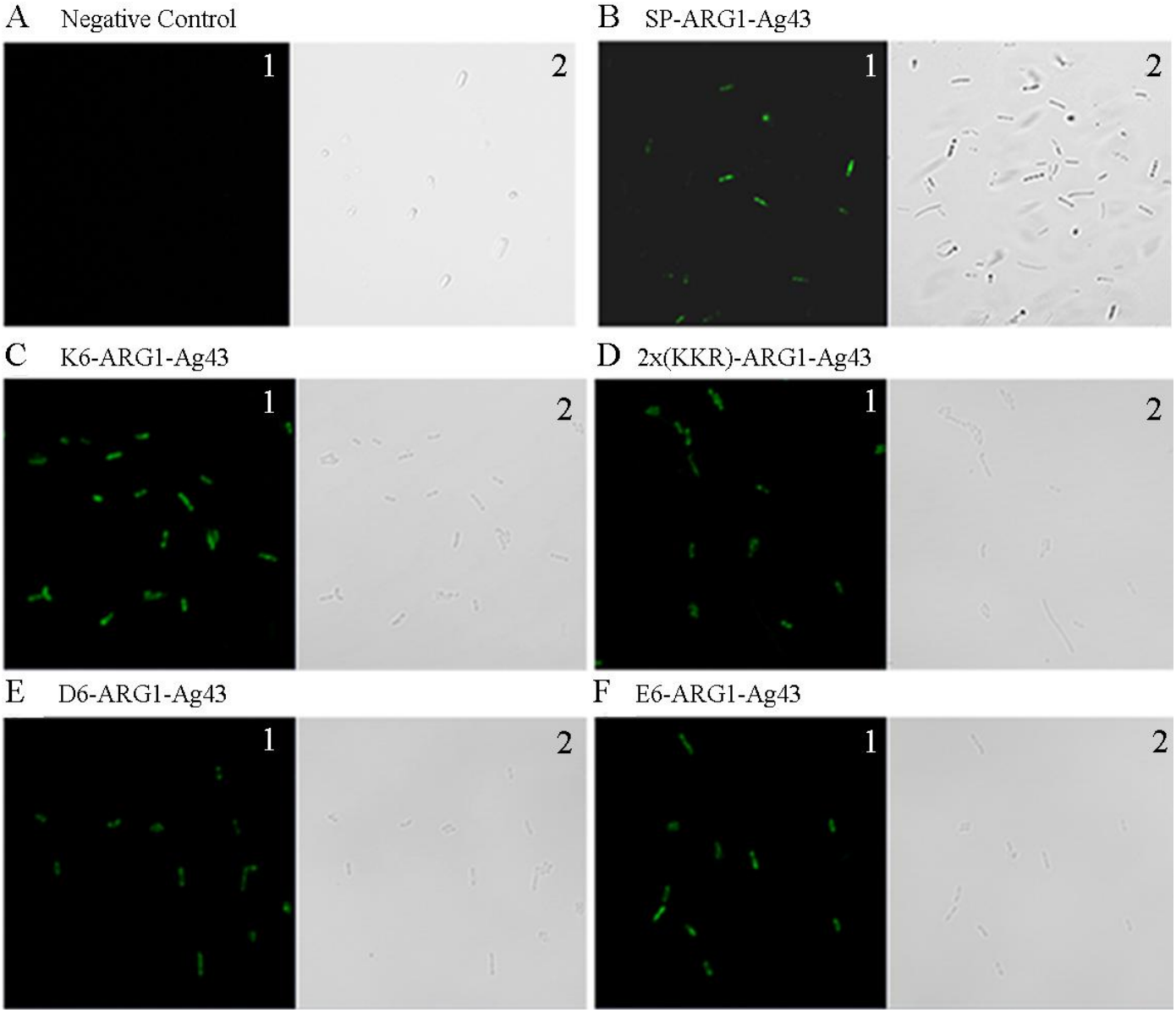

Figure 6. Fluorescence microscope assay of different engineered Ag43 Display System. Strains harboring different recombinant plasmids under fluorescence microscope. 1-6: pET23a-T empty vector, SP-ARG1-Ag43, K6-ARG1-Ag43, 2×(KKR)-ARG1-Ag43, D6-ARG1-Ag43, E6-ARG1-Ag43. 1: detecting of FITC signal, 2: the bright field.

display efficiency of nearly 2.6-fold and 1.6-fold, respectively (Figure 2). The results demonstrated that both of them could enhance the efficiency of surface display of human ARG1.The different recombinant *E. coli* cells labeled with FITC – conjugated fluorescent antibody were further confirmed by observation under the fluorescence microscope. As shown in Figure 6, solid fluorescence rods were observed in bacteria cells containing plasmid pET-23a-His6·tag/E6-ARG1-Ag43, pET-23a-His6·tag/K6-ARG1-Ag43, pET-23a-His6·tag/2×(KKR)-ARG1-Ag43, pET-23a-His6·tag/ARG1-Ag43 and pET-23a-His6·tag/D6-ARG1-Ag43 indicating that they could presence of human ARG1 on the surface of bacteria. No solid fluorescence rods were detected in the control cells harboring the empty pET23a-T construct.

Protein accessibility test

We also used the proteinase K accessibility test to further confirm that the human ARG1 was displayed on the bacteria surface. Proteinase K is too large to enter the

Table 4. Whole cell enzyme activity.

Cells	ProteinaseK-(U/mL± [d]SD)	ProteinaseK+(U/mL± SD)
SP-ARG1-ag43	0.208±0.0035	0.653±0.043
K6-ARG1-ag43	11.50±0.551	1.73±0.098
E6-ARG1-ag43	6.56±0.358	1.21±0.067
D6-ARG1-ag43	0.73±0.061	0.20±0.008
2×(KKR)-ARG1-ag43	10.90±0.086	1.83±0.052

Each value represents the mean of three independent measurements; [d]SD means the standard deviation of three independent determinations.

cell envelope, so it can only degrade the fusion proteins on the surface of cell membrane (Schumacher et al., 2012). The decreased arginase activity of the whole cells can reflect the degradation situation of cell surface presented proteins by Proteinase K. All in the contemplation, proteinase K accessibility test revealed the whole cell activity decreased approximately 85% for K6-ARG1-Ag43, 84% for 2×(KKR)-ARG1-Ag43, 88% for E6-ARG1-Ag43, 72% for D6-ARG1-Ag43 and 48% for SP-ARG1-Ag43, respectively (Table 4). Based on these results, it was confirmed that the fusion ARG1-Ag43 was successfully anchored on the outer membrane of the *E. coli* cells, with K6-ARG1-Ag43 allowing the highest proteinase K accessibility as well as the highest efficiency of surface display (Figure 2C).

Characteristics of displayed human ARG1 in *E.coli*

Whole cell ARG1 activity assay was performed using the Chinard reaction for further investigation (Chinard, 1952). Cells harboring vector pET23a-T used as a negative control and presented no arginase activity. The original Ag43 system displayed ARG1 presented activity of 0.69 U/mL (OD_{600}=1.0), while cells harboring the plasmid pET-23a-His6·tag/D6-ARG1-Ag43 and pET-23a-His6·tag/E6-ARG1-Ag43 were slightly enhanced to 1.22 U/mL (OD_{600}=1.0) and 8.38 U/mL (OD_{600}=1.0), respectively. However, cells harboring the plasmid pET-23a-His6·tag/K6-ARG1-Ag43 and pET-23a-His6·tag/2×(KKR)-ARG1-Ag43, could greatly enhance nearly 22-fold and 20-fold of the original Ag43 system which presented activity of 15.21 U/mL(OD_{600}=1.0) and 14.33 U/mL(OD_{600}=1.0), respectively (Figure 3B).

According to the above results, cells harboring the plasmidpET-23a-His6·tag/K6-ARG1-Ag43 showed the highest enzyme activity and surface display efficiency, so we further investigated its potential application in hydrolyzing of L-Arg to L-Orn. Based on the above described series experiments (Figures 4A and B), the optimum hydrolysis reaction was pH 10.0 and the temperature was 50°C. Different material ions had been added to reaction mixture to value the effects on the ARG1 catalytic activity (Figure 4C). Based on our results, Mn^{2+}, $Ni^{2+}$and Co^{2+} had slightly enhanced the enzyme activity, while Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, $Mg^{2+}$and Ca^{2+} significant decreased the enzyme activity, especially the high concentration. For example, reaction mixture containing 5 mM of Fe^{3+} or Cu^{2+} can inhibit enzyme activity, respectively (Figure 4C). To obtain the best catalytic efficiency, the concentration of Mn^{2+} had been further investigated. The result indicated that lower concentration of Mn^{2+} could increase the enzyme activity, while higher concentration inhibited enzyme activity, and the best result was obtained with the application of 3mM of Mn^{2+} (Figure 4D).

As the L-Orn had widely used in health care and pharmaceutical industries, the displayed ARG1 had the potential as a whole-cell catalyst for producing of L-Orn due to its high enzyme activity and simple purification of product. Further experimental results indicate that the cells contained plasmid pET-23a-His6·tag/K6-ARG1-Ag43; possessed high stability in batch reaction. After 10 repeated reaction cycles, the enzymes remained more than 35% of its initial activity under the optimum pH 10 and the temperature at 50°C,3 mM of Mn^{2+} (Figure 4E). Using the blank control as a reference, the product of L-Orn was identified by LC-MS, there did not had L-Arg peak appeared in LC-MS (Figure 7), showing that nearly 100% of L-Arg (200g/L) was completely hydrolyzed within 16 h.

DISCUSSION

Ag43 is one of superfamily of AT belonging to type V secretion system which was the widely used secretion system for surface display (Rutherford and Mourez, 2006). In previous works, the first AT was Neisseria gonorrhoeae IgA protease discovered by Jose et al. (1995). ATs had distinctive organization in three main features, including N-terminus signal peptide which belonging to sec-pathway mediated translocation across the inner membrane, followed the passenger domains was translocated to the outer membrane with the help of the C-terminus region called β-domain (Rutherford and

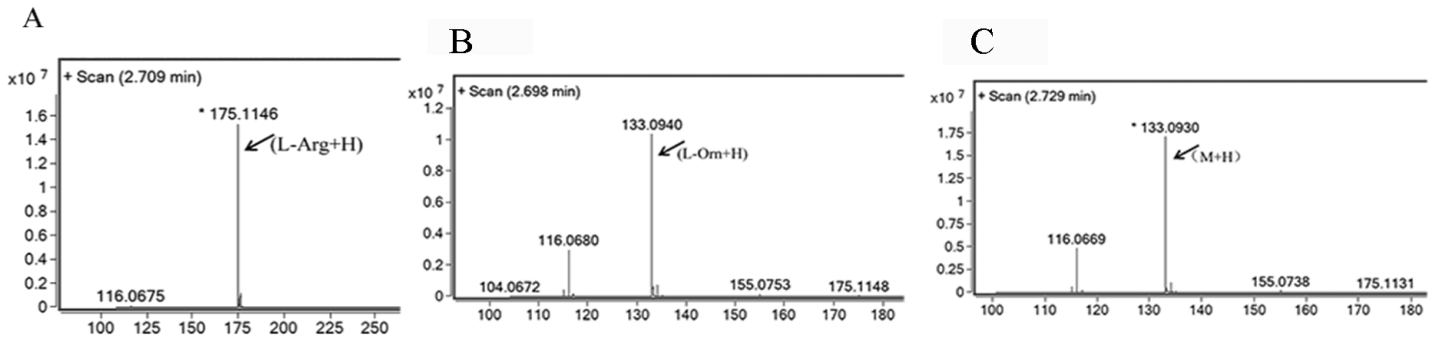

Figure 7. LC-MS analysis of the hydrolysis of L-Arg catalyzed by cells harboring the K6-ARG1-Ag43. **(A)** Cells harboring the plasmid pET23a-T-vector as the control. **(B)** L-Orn standard substance. **(C)** Cells harboring the recombinant plasmid K6-ARG1-Ag43 reaction supernatant.

Mourez, 2006). Many recombinant proteins had been tobe anchored with ATs for displaying various proteins or better understanding of mechanistic of ATs translocation (Rutherford and Mourez, 2006). However, the exact mechanism of the transport process remains enigmatic (Leo et al., 2012). According to numerous previous studies, the N-terminus signal peptide of Ag43 mediated passenger domain translocated through inner membrane into periplasmic space then translocated across outer membrane (Natale et al., 2008).

In research, human ARG1 had been successfully immobilized on the surface of *E. coli* through INP display system (Zhang et al., 2016). However, the surface display efficiency was relatively low as well as lower whole cell activity (the highest display efficiency was 14.8% and whole cell activity was 13.47 U/mL). In this study, four engineered charged polypeptides was used to replace the native signal peptide of Ag43 to analyze the efficiency of surface display of human ARG1. The results suggested that, the charged polypeptides had significantly influence on the surface display efficiency of ARG1. Briefly, 6×Lys, 6×Glu and 2×(KKR) could increase the surface display efficiency of human ARG1 for nearly 7.6-fold, 2.5-fold and 4.1-fold compared with native signal peptide of Ag43 system (Figures 2B-E), respectively. This new Ag43 display system (K6-ARG1-Ag43) had higher surface display efficiency (1.5-fold) and whole cell activity (1.1-fold) compared with our previous work (Zhang et al., 2016). These surprising effects of the charged polypeptides might be associated the N-terminal pI-specific directionality and their interactions with human ARG1 and β-domain. It was reported that the total translational efficiency of the proteins was based on the ΔG_{RNA} value of the N-terminal coding regions (Lee et al., 2011). The charged polypeptides of N-terminus sequence might influence the proteins translocation efficiency. Moreover, a short N-terminal polypeptide with a correct pI value and hydrophilicity could improve the secretion efficiency (De Keyzer et al., 2003). Meanwhile, the charged polypeptides could facilitate the protein in pre-fold form when translocation across the membrane. Otherwise, the interactions of 6×Lys, 6×Glu and 2×(Lys/Lys/Arg) with human ARG1 might also make contribution to the surface display efficiency of human ARG1 when used Ag43 display system.

Therefore, it is worth to note that that using the engineered ag43 display system, human ARG1 was successfully displayed on the surface of *E. coli.* Recombinant strains harboring the plasmid K6-ARG1-Ag43 presented the highest enzyme activity of 15.21 U/mL(OD_{600}=1.0) (Table. 4) as well as highest surface display efficiency (Figure 2C). In a simulated batch conversion reaction, the immobilized cells could hydrolyze L-Arg (200 g/L) to L-Orn within 16 h under the optimum pH 10.0 and temperature 50°C. As we know, human ARG1 need to form a trimmer to obtain its enzyme activity which was the main obstacle for its application. However, our results indicated that engineered Ag43 system can provide a new surface display system in Gram-negative bacteria for human ARG1. By coupling with FACS technology, surface-immobilized ARG1 might provide an alternative method for engineering human ARG1 in a high-throughput manner. At the same time, the immobilized human ARG1 can served as a whole-cell catalyst for producing L-Orn, which had a great potential in industrial application.

In summary, the charged polypeptides had significantly influence on the Ag43 surface display system. Although the detailed molecular mechanism is unclear, the results presented in this study provide new insights for displaying proteins of interest using the Ag43 display system.

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