Surface display of recombinant proteins on *Escherichia coli* by BclA exosporium of *Bacillus anthracis*

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**Abstract**

**Background:** The anchoring motif is one of the most important aspects of cell surface display as well as efficient and stable display of target proteins. Thus, there is currently a need for the identification and isolation of novel anchoring motifs.

**Results:** A system for the display of recombinant proteins on the surface of *Escherichia coli* was developed using the *Bacillus anthracis* exosporal protein (BclA) as a new anchoring motif. For the surface display of recombinant proteins, the BAN display platform was constructed in which a target protein is linked to the C-terminus of N-terminal domain (21 amino acids) of BclA. The potential application of BAN platform for cell surface display was demonstrated with two model proteins of different size, the *Bacillus* sp. endoxylanase (XynA) and monooxygenase (P450 BM3m2). Through experimental analysis including outer membrane fractionation, confocal microscopy and activity assay, it was clearly confirmed that both model proteins were successfully displayed with high activities on the *E. coli* cell surface.

**Conclusions:** These results of this study suggest that the strategy employing the *B. anthracis* BclA as an anchoring motif is suitable for the display of heterologous proteins on the surface of *E. coli* and consequently for various biocatalytic applications as well as protein engineering.

**Background**

Cell surface display allows expression of proteins or peptides on the surface of cells in a stable manner using the surface proteins of bacteria, yeast, or even mammalian cells as anchoring motifs [1-4]. This powerful tool has been used in a wide range of biotechnological and industrial applications, such as live vaccine development [5], peptide libraries screening [6,7], whole-cell catalysis [8], biosensor development [9,10] and environmental bioadsorption [11,12]. For the efficient display of recombinant proteins on a surface of host cells, various anchoring motifs have been developed, including OprF, OmpC, OmpX, and many others [12-14]. Although many successful results have been achieved, the use of current anchoring motifs did not always allow efficient display of all target proteins [3]. In cell surface display systems, successful protein display is highly dependent on the choice of the anchoring motif. Therefore, in this study, we decided to explore and develop an alternative cell surface display system for the expression and display of recombinant proteins.

The surface protein to be used as an anchoring motif should possess in general an efficient signal sequence to facilitate the translocation of a foreign protein through the inner membrane of the cell, a targeting signal for anchoring a foreign protein to the surface of the cell in a stable manner, and accommodating foreign proteins or peptides of various sizes. Furthermore, the fusion protein should be expressed in large amounts [2-4]. Here, we developed a cell surface display system using the *B. anthracis* BclA as a potential anchoring motif. The BclA is an exosporium protein, a hair-like protein surrounding the *B. anthracis* spore. The *B. anthracis* BclA proteins were found to possess conserved amino acid...
sequences (red-colored in Additional file 1: Figure S1a) in the N-terminal domain (NTD), C-terminal domain (CTD), and central (GPT)$_3$GDTGTT triplet repeating region (blue-colored in Additional file 1: Figure S1a) making a collagen-like structure (Figure 1a) [15,16]. In this work, we developed a protein display system (BAN platform) using the NTD (21 amino acids) of BclA as an anchoring motif and its display efficiencies were examined with two model recombinant proteins, a relatively small Bacillus sp. endoxylanase (XynA, 21.2 kDa) and a much bigger Bacillus megaterium monoxygenase (P450 BM3m2, 120 kDa).

**Results**

**Development of a surface display system with BclA**

The native BclA contain 19-residue amino terminal peptide, but this peptide is proteolytically removed during sporulation and the remaining mature BclA is attached to the surface of the developing forespore [15,16]. The mature BclA protein consists of three parts: NTD, CTD and the central domain, which contains 1 ~ 8 repeating regions of ‘(GPT)$_3$GDTGTT triplet sequence’ (Figure 1a and Additional file 1: Figure S1) [15,16]. The central domain looks like a mammalian collagen protein (it is also called ‘collagen-like region,’ CLR) and, according to the repeating number of CLR, the BclA can be of different sizes (253 ~ 445 amino acids). Each domain has its own unique independent function. In addition, the truncated form of each domain shows different levels of expression and localization on the membrane [8,16,17]. Thus, in order to develop the most efficient display system based on the B. anthracis BclA, we examined three different display systems using the three different motifs (BAN, BANC, and BAF) of BclA as anchoring motifs as shown in Figure 1b. The BAN system contains only the NTD (21 amino acids) without the 19-residue amino-terminal peptide of BclA, and the BANC system contains both NTD and CTD without the central CLR (total 178 amino acids). Finally, the BAF system contains a mature form of full-length BclA from B. anthracis RA3 strain (NCBI accession no. CAD56878.1, 233 amino acids). In each system, the target protein (Bacillus sp. TG43 lipase) was fused to the C-terminus of each anchoring motif, and gene expression was controlled under the IPTG-inducible tac promoter (P$_{tac}$). Comparison of gene expression in three systems suggested that the BAN expression platform (pTJ1-BAN) allowed significantly higher gene expression, while other expression systems (BANC and BAF) showed rather poor expression levels (Additional file 2: Figure S2). Even though much higher production level could be achieved with the BAN-fused system, the localization of BAN-fused lipase on cell surface gave multiple varying results during the repeated experiments. Thus, the display of other recombinant proteins instead of lipase was studied using the BAN platform as a display system in further experiments.

**Display of endoxylanase on the E. coli cell surface**

To demonstrate the potential of BclA as an anchoring motif for cell surface display, the Bacillus sp. endoxylanase (~21.2 kDa), which can hydrolyze xylan to xylooligosaccharides (xylose, xylobiose, xylotriose, etc.) [18], was examined. For the production of BAN-fused endoxylanase, two plasmids, pTJ1-BAN-XynA and pTJ1-pelB-BAN-XynA, were constructed in which the BAN motif was fused to XynA without or with the PelB signal peptide, respectively. As a negative control, pTJ1-pelB-XynA, which does not contain the BAN anchoring motif but containing the PelB signal peptide for the periplasmic production of XynA, was also constructed. Recombinant E. coli strains harboring pTJ1-pelB-XynA, pTJ1-BAN-XynA, and pTJ1-pelB-BAN-XynA were cultivated, and the localization of endoxylanase on the cell surface was analyzed by SDS-PAGE and western blotting. The BAN-linked endoxylanase was clearly detected in the total lysate and outer membrane fractions of both E. coli cells harboring pTJ1-BAN-XynA and pTJ1-pelB-BAN-XynA (Figure 2a). However, when the PelB signal peptide without the BAN motif (pTJ1-pelB-XynA) was used, endoxylanase was not detected in the membrane fraction, but was detected in the periplasmic fraction only.
The production and localization of BAN-fused endoxylanase on membrane fractions were also clearly confirmed by western blotting (Figure 2b). In addition, localization of endoxylanase could be confirmed by confocal microscopy. After cultivation, cells were labeled with the FITC-conjugated anti-FLAG antibody probe, which can recognize the FLAG tag linked to C-terminus of endoxylanase. E. coli cells harboring pTJ1-BAN-XynA and pTJ1-pelB-BAN-XynA showed the strong fluorescence, while E. coli harboring pTJ1-pelB-XynA did not show any fluorescence signal (Figure 3). This means that the BAN anchoring motif mediated the secretion and localization of XynA on the surface of E. coli independent of the signal peptide.

The specific endoxylanase activities of whole cells were determined using beechwood xylan as a substrate. Immediately after mixing with xylan, E. coli harboring pTJ1-BAN-XynA also showed a significant increase in the concentration of reducing sugars compared with other controls (E. coli cells harboring pTJ1-pelB-XynA or pTJ1-pelB-BAN-XynA) (Figure 4). This result suggests that the endoxylanase was successfully anchored to outer membrane by fusion with BAN anchoring motif, and the displayed endoxylanase showed high activity. The use of PelB signal peptide with the BAN anchoring motif (pTJ1-pelB-BAN-XynA) also allowed the secretion and localization of the BAN-fused endoxylanase. However, its display efficiency and activity were relatively lower than those obtained with the BAN motif only.
Thus, the use of signal peptide is not necessary in the BAN display platform. The localization of excess proteins on outer membrane might cause problems in cell wall integrity, and consequently result in cell lysis and possible release of anchored proteins into the culture medium. Thus, the contamination of endoxylanase in the culture supernatant was analyzed during the display of endoxylanase on the surface. After cultivation of *E. coli* harboring pTJ1-BAN-XynA, the presence of endoxylanase on the surface and culture medium were analyzed by SDS-PAGE followed by western blotting analysis. The BAN-fused endoxylanase was clearly detected in the total cell lysate and membrane fractions, but not in the supernatant fraction (Additional file 3: Figure S3). This result indicates that BAN-fused endoxylanases were displayed on the surface and were not released into the culture medium. The specific endoxylanase activities in whole cells and culture supernatant were determined as described above. A low level of activity from the culture supernatant of *E. coli* (pTJ1-BAN-XynA) was obtained, but it was much lower than that of the whole-cell suspension (Additional file 4: Figure S4). This result also indicates that the BAN-fused endoxylanase was successfully anchored on the cell surface and was stably maintained without cell lysis.

Display of monooxygenase on the *E. coli* cell surface

To demonstrate the general use of the BAN anchoring motif for cell surface display, cytochrome P450 monooxygenase variant (P450 BM3m2) from *B. megaterium* was also examined; this protein is much bigger (ca. 120 kDa) than endoxylanase. After flask cultivation of *E. coli* XL1-Blue harboring pTJ1-BAN-BM3, localization of the BAN-fused P450 BM3m2 on the cell surface was confirmed by SDS-PAGE. The band of P450 BM3m2 (~120 kDa) was clearly detected on the Coomassie-brilliant blue stained SDS-PAGE gel (Figure 5a). However, with *E. coli* harboring pTJ1-BM3 in which the BAN motif was not used, P450 BM3m2 was produced in the cytoplasm only and was not detected in the outer membrane fraction. The localization of P450 BM3m2 on the cell surface was also confirmed by confocal microscopy. After cultivation and labeling with the FITC-conjugated anti-FLAG antibody probe which can recognize the FLAG tag linked to C-terminus of P450 BM3m2. *E. coli* harboring pTJ1-BAN-BM3 clearly exhibited strong fluorescent signals, but *E. coli* harboring pTJ1-BM3 did not show any fluorescent signals (Figure 5b). The activity of monooxygenase (P450 BM3m2) on the surface of *E. coli* cells was verified using a specific activity assay. In this activity assay, the catalytic conversion of 7-ethoxycoumarin to 7-hydroxycoumarin requires the regeneration of oxidative monooxygenase, and NADPH was externally supplied for this regeneration purpose. However, it is known that NADPH cannot penetrate the cell membrane [19], so only the monooxygenase displayed on the cell surface can utilize the supplied NADPH and continue the oxidative reaction. However, cytoplasmic monooxygenase cannot be regenerated due to the absence of supplied NADPH, and it cannot continue the reaction. The high activity of monooxygenase indicates the localization of monooxygenase on the cell surface.
Discussion
Microbial cell-surface display systems can be used in a wide range of applications as described earlier. The display of active enzymes has been intensively pursued due to its potential use as a whole-cell biocatalyst in the fields of pharmaceuticals, fine chemicals and agrochemicals production. To date, several cell-surface display systems have been developed for the expression of polypeptides or proteins on the surface of *E. coli* and, as an anchoring motif, outer membrane proteins such as OmpC, OmpX, maltoprotein LamB, the outer membrane protein S, lipoprotein TraT and many others, have been employed, which were fused to the protein of interest [3]. In most of the surface display systems that use outer membrane proteins as anchoring motifs, target proteins are fused to the anchoring motif via a sandwich fusion format. This results in minimal destabilization of the anchoring motif on the outer membrane, and improved efficiency of surface display. In an alternative, which includes the OmpX case and has been called the circularly permutated strategy, both the N and C termini are presented on the external cell-surface [14]. However, in most cases, the passengers displayed have been limited primarily to short peptides or polypeptides. In the present study, the *B. anthracis* exosporal protein BclA was examined as a new anchoring motif. The C-terminal fusion strategy was employed to display foreign proteins. In this display format, both proteins (endoxylanase and monooxygenase) were successfully displayed with high activities. These results suggest that the C-terminal deletion-fusion strategy employing the *B. anthracis* BclA is suitable for the display of heterologous polypeptides and proteins, which should prove useful in a wide range of applications. Particularly, the successful display of monooxygenase (P450 BM3m2), which has much bigger size than the average size of passenger proteins [3], was very promising. In general, most cell display systems have size limitations in regards to the protein to be displayed, and most systems are only suitable for peptides or relative small polypeptides (below 50 kDa). So far, there is only one successful case for the display of P450 on a bacterial cell surface, where an ice-nucleation protein (INP) was used as an anchoring motif [19]. When other well-known anchoring motifs including OmpC and OmpX were tested, such a big-size enzyme (P450 BM3m2) could not be displayed (data not shown). The successful display of P450 using the BAN anchoring motif clearly indicates the potential of the BAN anchoring motif to accommodate many different passenger proteins.

Although two model proteins of different sizes could be successfully displayed on the cell surface using the BclA anchoring motif, the mechanisms for the secretion and anchoring of BclA and BclA-fused proteins are unknown at this time. In general, the protein secretion into periplasm and cell surface including cell surface display requires a signal peptide. The native BclA protein synthesized in the mother cell is attached to the surface of the forespore; the 19-residue amino-terminal peptide is proteolytically removed and the next 20–40 amino acids (NTD) are used for anchoring. Also, it is known that the anchoring of the mature BclA protein on the surface of *B. anthracis* requires a few specific exosporium receptor proteins (i.e., BxpB or its homologue ExsFB) [20-22]. In the display system reported here, only the NTD (21 amino acids) after the first 19 amino acids of BclA was employed. To our knowledge, the receptor proteins reported for *B. anthracis* are not produced in *E. coli*. As demonstrated with two model proteins (endoxylanase and P450 BM3m2), their fusions with the BAN motif resulted in their successful secretion and localization onto the cell surface, which was clearly confirmed by several experiments including membrane protein fractionation, confocal microscopy, and activity assay. The use of the well-known PelB signal peptide for the secretion of BAN-fused proteins also allowed the localization of proteins on the cell surface of *E. coli*, but the efficiencies were relatively lower than that obtained with the signal peptide-free system (Figures 2, 3 and 4).
Thus, it will be an interesting future study to decipher the detailed mechanisms for such signal peptide-free secretion using the BAN system. However, this is not the only case reported. The ice nucleation protein (INP) of *Pseudomonas syringae* has been used as a great anchoring motif for the cell surface display of various proteins in *E. coli* and other gram negative bacteria. The INP and its fusion proteins do not require any signal peptide for their secretion and anchoring on the cell surface [8,17,23]. Interestingly, the BclA protein has structural similarity to INP which also comprises three domains (NTD, CTD and CLR). Thus, although there is no proof that INP and BclA could mediate membrane transport and cell attachment in a similar way, the mechanism of BAN-based signal peptide-free secretion might be similar to that of INP. Further studies are needed to understand the mechanism.

**Conclusions**

We developed a new cell surface display platform based on the BclA of *B. anthracis* as an anchoring motif. The C-terminal fusion of two model proteins to BclA (especially BAN) allowed their successful secretion and surface display with high efficiency and stability. Although only two proteins (21 and 120 kDa) were examined, the results seem to indicate the versatility of this display system capable of secreting and displaying proteins of different sizes; this is an important advantage of the BAN anchoring motif. Furthermore, both enzymes displayed using the BAN system, endoxylanase (XynA) and monooxygenase (P450 BM3m2), showed high activities and stabilities (as examined for xylanase). By displaying different enzymes of interest using our system, it will be possible to develop cost-effective bio catalytic systems in the fields of pharmaceuticals, fine chemicals, agrochemicals and other demanding industries.

**Materials and methods**

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this study are summarized in Table 1. All *E. coli* cells were cultivated in Luria-Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of NaCl) supplemented with 50 μg/mL of ampicillin (Ap) at 37°C and 200 rpm. For the display of endoxylanase, cells were induced at an OD600 of 0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, all cells were further cultured for 4 h and then harvested by centrifugation (10,000 × g for 10 min at 4°C) for further analysis. For the display of P450 BM3m2, *E. coli* cells were induced at the same cell density with 0.4 mM IPTG. In addition, 1 mM of thiamin and 0.5 mM of δ–aminolevulinic acid (Sigma-Aldrich, St. Louis, MO) as a heme precursor were added into each culture at the same time with

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|-------------------------|---------------------|
| **Strains** | | |
| *E. coli* JM109 | FtrA D36 proA + 8 + lacIqΔ(lacZ)M15/Δ(lac-proAB) glnI44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17 | New England Biolabs® |
| *E. coli* XL1-Blue | recA1 endA1 gryA96 thi-1 hsdR17 supE44 relA1 lac (F’ proAB lacIqZΔM15 Tn10 Tetr). | Stratagene® |
| **Plasmids** | | Pharmacia® |
| pTrc99A | 4.17 kb, bla, trc promoter | [24] |
| pTac99A | 5.68 kb, pTrc99A derivative; tac promoter | This study |
| pTJ1-BAN | 5.76 kb, pTac99A derivative; N-terminal of bclA | This study |
| pTJ1-BANC | 6.23 kb, pTac99A derivative; N- & C-terminal of bclA | This study |
| pTJ1-BAF | 6.45 kb, pTac99A derivative; bclA | This study |
| pKUX4 | pUC19 containing endoxylanase gene (xynA) | [18] |
| pTJ1-BAN-XynA | 6.8 kb, pTJ1-BAN derivative; BAN-fused xynA with His6 tag (N-terminus) and FLAG tag (C-terminus) | This study |
| pTJ1-pelB-BAN-XynA | 6.36 kb, pTJ1-BAN derivative; pelB signal peptide, BAN-fused xynA with FLAG tag (C-terminus) | This study |
| pTJ1-pelB-XynA | 6.28 kb, pTJ1 derivative; pelB signal peptide, xynA with FLAG tag (C-terminus) | This study |
| pTJ1-BAN-BM3 | 9.0 kb, pTJ1-BAN derivative; BAN-fused *B. megaterium* monooxygenase (P450-BM3m2) gene with His6 tag (N-terminus) and FLAG tag (C-terminus) | This study |
| pTJ1-BM3 | 8.9 kb, pTJ1-BAN derivative; *B. megaterium* monooxygenase (P450-BM3m2) with FLAG tag (C-terminus) | This study |

*New England Biolabs, Beverly, MA.
Stratagene, Santa Clara, CA.
Pharmacia Biotech, Uppsala, Sweden.*
IPTG induction. After further cultivation at 30°C for 6 h and 150 rpm, cells were harvested by centrifugation at 6,000 rpm for 10 min for further analysis.

Plasmids and DNA manipulation
The full gene of the \textit{B. anthracis} RA3 (NCBI accession no. CAD56878.1) \textit{bclA} was synthesized by the \textit{de novo} gene synthesis method (GENEMAKER\textsuperscript{TM}; Blue Heron Biotechnology, Bothell, WA). All PCR primers used in this study are listed in Table 2. DNA amplification of the \textit{bclA} gene was performed with primers P1 and P2. The PCR product was digested with EcoRI and Xhol restriction enzymes, and cloned into pTac99A \cite{24} to yield pTJ1-BAF, which contained the full gene of BclA. Two truncated forms of the \textit{bclA} gene, which contained the N-terminal region (BA-N) or N- and C-terminal fused region (BA-NC) without the repeated region (BA-RD), were synthesized by PCR with the primers P1 and P3, and P4 and P5, respectively. After digestion with EcoRI and Xhol restriction enzymes, each PCR product was cloned into pTac99A to yield pTJ1-BAN and pTJ1-BANC, respectively. The endoxylanase gene (\textit{xynA}) of \textit{Bacillus} sp. was amplified from pKJX4 \cite{18} as a template using standard procedures \cite{26}. All DNA manipulations, including restriction digestion, ligation, and agarose gel electrophoresis, were carried out using standard procedures \cite{26}.

Fractionation of outer membrane proteins
Outer membrane proteins were prepared and analyzed as previously described \cite{27,28}. Briefly, after washing cells with 0.5 mL of 10 mM Na\textsubscript{2}HPO\textsubscript{4} buffer (pH 7.2) twice, cells were disrupted by three cycles of sonication (each for 20 s at 15% of maximum output; High-Intensity Ultrasonic Liquid Processors, Sonics & Material Inc., Newtown, CT). After quick centrifugation (12,000 \( \times \) g for 2 min) to remove partially disrupted cells, membrane proteins and the lipid layer were isolated by

Table 2 Oligonucleotides used for PCR amplification in this study

| No. | Primer sequences (5' → 3')\textsuperscript{a} | Gene to be amplified | Source or references |
|-----|---------------------------------------------|---------------------|---------------------|
| P1\textsuperscript{b} | GGAATTCTAGCACCACCAACCACACCACGGCATTTGACCTAATCCTT | Full \textit{bclA} gene | \textit{B. anthracis} RA3 |
| P2 | AGTCTAGACTCCAGCTAGCCCCGGGGTAGGAGGATATAGGG | Truncated \textit{bclA} gene (NTD) | \textit{B. anthracis} RA3 |
| P3 | CCACCATTTACCTTCTCTACGCCCTACGGACTAGGCTTT | Truncated \textit{bclA} gene (NTD + CTD) | \textit{B. anthracis} RA3 |
| P4 | AAGTCTAGATGGGCCGTTAGGAAGGGAATAATGCGG | \textit{Bacillus} sp. Endoxylanase (\textit{xynA}) | \cite{18} |
| P5 | CGCTAGACTCCAGCTACGGCCCAGCAGCACTTTCTAACAA | \textit{Bacillus} sp. Endoxylanase (\textit{xynA}) | \cite{18} |
| P6 | CAGCGTTAGCGTGGCAGCAGATGTAGGG | \textit{B. anthracis} RA3 |
| P7 | CCAGCTATTTTGTACATGTCATCTTTATAATCCACACTGTACATTGAGACTTC | \textit{B. anthracis} RA3 |
| P8 | AGAAATTCTAGAATCCCCTATTGCTACCGAGCCGGGATTGATTTACATTATCGGCGGCAA | \textit{B. anthracis} RA3 |
| P9 | ATGTTGTTACCTGGGGCCACCGGGCCATTGGCGCCACAGATATCTGGC | \textit{B. anthracis} RA3 |
| P10 | AGAAATTCTAGAATCCCCTATTGCTACCGAGCCGGGATTGATTTACATTATCGGCGGCAA | \textit{B. anthracis} RA3 |
| P11 | TGCTAGCCGCGGGGTT | \textit{B. anthracis} RA3 |
| P12 | GGCGTACATGACAAATAGAAGGATGCCTCAGGC | \textit{B. anthracis} RA3 |
| P13 | GCCCATGCTATTTTGTACATGTCATCTTTATAATCCACCGCAGATGTAGGG | \textit{B. anthracis} RA3 |
| P14 | GGAATTCTAGCAATATAGAAGAATGCTCAGGCC | \textit{B. anthracis} RA3 |

\textsuperscript{a} Restriction enzyme sites are shown in bold.
\textsuperscript{b} The sequence for six histidines is shown in italic.
centrifugation at 12,000 ×g for 30 min at 4°C and then, pellets were resuspended in 0.5 mL of 10 mM Na2HPO4 buffer (pH 7.2) and 0.5% (w/v) sarcosyl solution. After incubation at 37°C for 30 min, an insoluble pellet containing outer membrane proteins was obtained by centrifugation at 12,000 ×g for 30 min at 4°C. After washing the insoluble pellet with 10 mM Na2HPO4 buffer (pH 7.2), the outer membrane proteins were resuspended in 50 μL of TE buffer (pH 8.0).

**SDS-PAGE and western blotting analysis**

Protein samples were analyzed by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. For immunodetection of the FLAG-tag fused protein, a monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich) and goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (Invitrogen, Carlsbad, CA) were used. An ECL kit (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare) was used for signal detection.

**Measurement of endoxylanase activity**

Endoxylanase activity was measured using the 3′,5′-dinitrosalicicylic acid (DNS) method [29]. After cultivation, cells were washed twice with 1× phosphate-buffered saline (PBS), and then mixed with 2% (w/v) beech wood xylan solution. The reaction mixture was incubated at 37°C, and the supernatant of the mixture was sampled at time intervals by centrifugation for 10 min at 12,000 ×g and room temperature. The supernatant samples were mixed with 3 times the volume of a DNS solution containing 7.5 g 3,5-dinitrosalicicylic acid (DNS), 14 g NaOH, 216.1 g Rochelle salt, 5.4 mL phenol and 5.9 g Na2S2O5 per liter. After boiling for 10 min, samples were cooled to room temperature for 5 min, and then the absorbance of the reactant was detected by a spectrophotometer at 550 nm. One unit (U) of endoxylanase activity was defined as the amount of enzymes capable of producing 1 μmol of reducing sugar per min. The specific activity was defined as the endoxylanase activity for the amount of cells with an OD600 of 2.0.

**Measurement of monoxygenase (P450 BM3m2) activity**

After cultivation, cells were harvested by centrifugation at 6,000 rpm for 10 min. Cell pellets were washed twice with 100 mM potassium phosphate buffer (pH 7.4) and the cells were concentrated in the same buffer to a calculated final OD600 of 100. The activity of the displayed P450 was determined by analyzing the dealkylation of 7-epoxycoumarin to 7-hydroxycomuarin as described previously [25]. The prepared cells were mixed with 7-ethoxycoumarin (1 mM) and NADPH (0.5 mM) in 100 mM potassium phosphate buffer (pH 7.4). The reaction was performed at room temperature for 30 min, and the product (7-hydroxycomuarin) was quantified using a multi-well plate fluorometer (VICTOR ×3, Perkin-Elmer, Waltham, MA) with excitation wavelength at 405 nm and emission wavelength at 460 nm.

**Fluorescence microscopy**

After cultivation, cells (1 mL) were washed with 1× PBS and resuspended in 1× PBS supplemented with 3% (w/v) bovine serum albumin (BSA; Sigma-Aldrich). The cells were first incubated with the rabbit anti-FLAG probe antibody conjugated with FITC (Invitrogen) at a dilution of 1:250 for 1 h. Cells were washed 2 times with a PBS solution to remove the unbound probes. Finally, cells were mounted on a poly-L-lysine coated microscopic slide and examined by confocal microscopy (Carl Zeiss LSM510 META, Jena, Germany). Samples were excited by a 488 nm argon laser, and images were filtered by a long pass 505 nm filter. Images were acquired with Carl Zeiss LSM 510 software (version 4.2.rk).

**Additional files**

- **Additional file 1:** Figure S1. Multiple alignments of several BclA proteins and schematic representation of the BclA protein consisting of GXX triplet motifs.
- **Additional file 2:** Figure S2. SDS-PAGE analysis for the expression of lipase (Lip1) in three different BclA anchoring systems.
- **Additional file 3:** Figure S3. Display of endoxylanase on the cell surface.
- **Additional file 4:** Figure S4. Endoxylanase activity.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

TJP designed and performed most experiments, and analyzed data and drafted the manuscript. NSH participated in the display of BclA cloning, and SSY and JHP participated in the display of BclA cloning, and analyzed data and reviewed the manuscript. All authors have read and approved the final manuscript.

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References

1. Cornelis P: Expressing gene in different Escherichia coli compartments. Curr Opin Biotechnol 2000, 11:450–454.

2. Georgiou G, Stathopoulos C, Daugherty PS, Nayak AR, Iverson BL, Curtiss R: Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. Nat Biotechnol 1997, 15:29–34.

3. Lee SY, Choi JH, Xu Z: Microbial cell surface display. Trends Biotechnol 2003, 21:45–52.

4. Stahl S, Ulhen M: Bacterial surface display: trends and progress. Trends Biotechnol 1997, 15:185–192.

5. Lee JS, Shin KS, Pan JG, Kim CJ: Surface-displayed viral antigens on Salmonella carrier vaccine. Nat Biotechnol 2000, 18:645–648.

6. Martineau P, Charbit A, Leckier C, Werts C, O’Callaghan D, Hofnung M: A genetic system to elicit and monitor anti-peptide antibodies without peptide synthesis. Biotechnology 1991, 9:170–172.

7. Boder ET, Wittrup KD: Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol 1997, 15:553–557.

8. Jung HC, Lebeault JM, Pan JG: Surface display of Zymomonas mobilis levansucrase by using the ice-nucleation protein of Pseudomonas syringae. Nat Biotechnol 1998, 16:576–580.

9. Ghiloni JK, Drew PD, Porter AJ: Bacterial surface display of an anti-pollutant antibody fragment. Lett Appl Microbiol 1999, 28:350–354.

10. Shibasaki S, Ueda M, Ye K, Shimizu K, Kamawasa N, Osumi M, Tanaka A: Creation of cell surface-engineered yeast that display different fluorescent proteins in response to the glucose concentration. Appl Microbiol Biotechnol 2001, 57:528–533.

11. Sousa C, Kotbha P, Rumil T, Celba A, de Lorenzo V: Metalloadsorption by Escherichia coli cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB. J Bacteriol 1998, 180:2280–2284.

12. Xu Z, Lee SY: Display of polyhistidine peptides on the Escherichia coli cell surface by using outer membrane protein C as an anchoring motif. Appl Environ Microbiol 1999, 65:5142–5147.

13. Lee SH, Lee SY, Park BC: Cell surface display of lipase in Pseudomonas putida KT2442 using OprF as an anchoring motif and its bio catalytic applications. Appl Environ Microbiol 2005, 71:8581–8586.

14. Rice JJ, Scholt A, Bessette PH, Bouwlaire KT, Daugherty PS: Bacterial display using circularly permuted outer membrane protein OprX yields high affinity peptide ligands. Protein Sci 2006, 15:825–836.

15. Synveste P, Couture-Tosi E, Mock M: A collagen-like surface glycoprotein is a structural component of the Bacillus anthracis exosporum. Mol Microbiol 2002, 45:169–178.

16. Synveste P, Couture-Tosi E, Mock M: Polymorphism in the collagen-like region of the Bacillus anthracis BcIA protein leads to variation in exosporum filament length. J Bacteriol 2003, 185:1555–1563.

17. Jung HC, Park JH, Park SH, Lebeault JM, Pan JG: Expression of carboxymethylcellulase on the surface of Escherichia coli using Pseudomonas syringae ice nucleation protein. Enzyme Microb Technol 1998, 22:348–354.

18. Jeong KJ, Park IF, Kim MS, Kim SC: High-level expression of an endo-xylanase gene from Bacillus sp. In Bacillus subtilis DB104 for the production of xylooligosaccharide from xylan. Appl Microbiol Biotechnol 1998, 50:113–118.

19. Yim SK, Kim DH, Jung HC, Pan JG, Kang HS, Ahn T, Yun CH: Surface display of heme- and diflavin-containing cytochrome P450-BM3 in Escherichia coli: a whole cell biocatalyst for oxidation. J Microbiol Biotechnol 2010, 20:713–717.

20. Tan L, Turnbough CL Jr: Sequence motifs and proteolytic cleavage of the collagen-like glycoprotein BcIA required for its attachment to the exosporium of Bacillus anthracis. J Bacteriol 2010, 192:1259–1268.

21. Steichen CT, Kearney JF, Turnbough CL Jr: Characterization of the exosporum basal layer protein Bsp8 of Bacillus anthracis. J Bacteriol 2005, 187:5866–5876.

22. Synveste P, Couture-Tosi E, Mock M: Contribution of ExsFA and ExsFB proteins to the localization of BcIA on the spore surface and to the stability of the Bacillus anthracis exosporum. J Bacteriol 2005, 187:5122–5128.

23. Li Q, Yan Q, Chen J, He Y, Wang J, Zhang H, Yu Z, Li L: Molecular characterization of an ice nucleation protein variant (inaQ) from Pseudomonas syringae and the analysis of its transmembrane transport activity in Escherichia coli. Int J Biol Sci 2012, 8:1097–1108.

24. Park SJ, Lee SY: Efficient recovery of secretory recombinant protein from protease negative mutant Escherichia coli strains. Biotechnol Techn 1998, 12:815–818.

25. Lee SH, Kwon YC, Kim DM, Park CB: Cytochrome P450-catalyzed C-dealkylation coupled with photochemical NADPH regeneration. Biotechnol Bioeng 2013, 110:389–390.

26. Sambrook J, Russell D: Molecular cloning: a laboratory manual. 3rd edition. New York: Cold Spring Harbor Laboratory Press; 2001.

27. Filip C, Fletcher G, Wulf JL, Earhart CF: Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J Bacteriol 1973, 115:717–722.

28. Lee SH, Choi J, Park SJ, Park SY, Park BC: Display of bacterial lipase on the Escherichia coli cell surface by using FadL, as an anchoring motif and use of the enzyme in entanoselective biocatalysis. Appl Environ Microbiol 2004, 70:5074–5080.

29. Chang CC, Ryu DOY, Park CS, Kim JY: Enhancement of rice R-amylase production in recombinant Yarrowia lipolytica. J Ferment Bioeng 1997, 84:421–427.

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