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RESEARCH PAPER

New Bacterial Surface Display System Development and Application Based on *Bacillus subtilis* YuaB Biofilm Component as an Anchoring Motif

Daeun Kim, Wooil Kim, and Junehyung Kim

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Abstract Bacterial surface display system has been adopted in various biotechnological applications. In the case of *Bacillus subtilis*, most of the studies have been developed using spore based surface display system utilizing the inherent rigidity of spore against heat, alkali, and shear stress. But, spore harvest, purification and separation need additional cost and labor. To eliminate this procedure and to use the gram-positive nature of *B. subtilis*, YuaB, which is one of the major *B. subtilis* biofilm components and locates in the cell wall, based cell surface display system, is developed. P43 promoter driven overexpression of YuaB-His_6 tag does not hamper bacterial cell growth and promoted biofilm formation of recombinant strain. Flow cytometry of recombinant strain and its protoplast using FITC-Anti His_6 antibody, verified that YuaB locate in plasma membrane and protrude to the outside of cell wall, which means YuaB can be used as very efficient anchoring motif. Using surface expressed YuaB-His_6 tag, removal of divalent metal ion, Cu^{2+} and Ni^{2+}, was tried to test its possibility for the environmental application of developed system.

Keywords: YuaB, flow cytometry, *Bacillus subtilis*, bacterial surface display system, protoplast

Daeun Kim, Wooil Kim, Junehyung Kim*
Department of Chemical Engineer, Dong-A University, Busan 49315, Korea
Tel: +82-200-7719; Fax: +82-51-200-7728
E-mail: june0302@dau.ac.kr

Junehyung Kim
Center for Sliver-Targeted Biomaterials, Brain Busan 21 Plus Program, Graduate School, Dong-A University, Busan 49315, Korea

1. Introduction

With increasing interest of global warming and environment, bioengineering has emerged as a promising alternative of chemical engineering industry. Application of bioengineering techniques can prevent formation of harmful byproducts, which is a main problem of chemical production process [1,2]. In bioengineering, many kinds of microorganisms have been widely used and studied in various field such as chemical [3,4], biopolymer [5,6], pharmaceutical [7,8], and nutraceutical [9]. Some of most popular microorganisms in biomanufacturing are *Escherichia Coli*, *Corynebacterium* species, *Bacillus* species, *Pseudomonas* species, *Clostridium* Species, and *Saccharomyces cerevisiae* [10].

Among numerous species, *Bacillus subtilis* has been considered as attractive bacterium for industrial application. *B. subtilis* is a gram-positive bacterium which has distinct features such as good growth on cheap carbon source, clear inherited backgrounds, having genetic manipulation method, robustness in large-scale fermentation, spore formation and biofilm formation [11]. In addition, *B. subtilis* has been designated as generally recognized as safe (GRAS) according to the World Health Organization (WHO) [12]. A number of bio-products were already produced with dynamic strategies of engineering *B. subtilis* including metabolic optimization and spore display.

Bacterial surface display system has been applied in various biotechnological and industrial fields such as vaccine, biocatalyst, and biosensor development [13]. Bacterial surface display system was first introduced in 1986 by Roland Freudl *et al*. [14]. OmpA, which is one of the major outer membrane proteins of *E. coli* K-12 and exposed to external environment, was used as an anchoring motif for surface display system [14]. Recently, displaying laccase, CotA, of *B. subtilis* which has enzymatic activity decomposing

synthetic dye, on *E. coli* surface has been reported [15]. In the case of *B. subtilis*, most of studies have been focused on development of spore surface display system. *B. subtilis* spore is composed of peptidoglycan layer called the cortex, multilayer protein coat, and an outer coat layer [16]. Under harsh conditions such as heat, pH, ultraviolet radiation, and chemical stress, *B. subtilis* enters into the resting phase of sporulation and survive for a long time [12]. Due to its attributes, spore display system has been recognized as most promising engineering technology.

However, spore surface display system has some defects. In order to use spore of *B. subtilis*, bacterial cells have to be cultivated at least 24 h, because *B. subtilis* forms spores when they are exposed to environmental stress, like lack of nutrients. Moreover, after 24 h cultivation, we need additional process to isolate spore from cell. Commonly, spores have been separated by density difference between spore and cell with some chemicals that can provide density gradient such as PEG (poly ethylene glycol) [17] and Renografin (sodium diatrizoate hydrate) [18]. Spore separation process is complicate and highly expensive process, also difficult to get pure and large amounts of spores. Although spore display system has some drawbacks, cell surface display system of *B. subtilis* has not been studied as much as spore display system developed. Few studies have been reported by using sortase and sortase substrate [19,20]. Sortase makes covalent bond between *B. subtilis* cell wall and sortase substrate, but specific mechanism of *B. subtilis* sortase system is not certainly revealed yet.

B. subtilis forms rugose pellicles called biofilm, which is the group of bacterial societies by sticking cells each other [21]. Biofilm of *B. subtilis* is composed of self-produced extracellular matrix including exopolysaccharide (EPS), proteins, and some nucleic acid [22,23]. Development of biofilm is strictly regulated by gene expression of extracellular matrix components. Under certain conditions, for instance, nutrient depletion, impaired respiration or surface adherence [24-26], *B. subtilis* switches from a planktonic to a sessile state and start to stimulate genetic expression regarding production of extracellular components. After expansion and maturation of biofilm, it continues development by secretion of self-produced extracellular matrix. As a result, extensive wrinkles are created at the surface of biofilm matrix. Ruggedness of biofilm provides several advantages for the bacterial society. By increasing surface-to-volume ratio, bacteria within the biofilm have a greater access to oxygen [24,27]. In addition, biofilm has well-defined channels, which can transport liquid through extracellular matrix [28]. Liquid flux is crucial for providing nutrients and disposal of wastes. Moreover, genetic differentiation and sporulation of *B. subtilis* depend on the location inside the biofilm matrix [29]. It means that biofilm brings heterogeneity of cellular phase of *B. subtilis*.

EPS and proteins comprise of structural biofilm [30]. EPS, one of the major molecules of biofilm, is controlled by the *espA-O* gene operon (eps) [29-31]. However, the specific structure of EPS has not been fully understood. A study regarding the role of eps has been reported as deficiency of resulted in formation of weak and fragile pellicles. Although, other matrix components support deficiency of EPS and EPS mutant can keep growing in extracellular matrix [30]. TasA and TapA are two important proteins, which are the products of three-gene operon *tapA-sipW-tasA* (*tapA* operon) [32]. Long intercellular amyloid fibers, which provide structural stability of biofilm, are formed by self-assembly of TasA protein [30,33]. This amyloid fibers anchor to the cell wall with the help of TapA [33]. Additionally, YuaB (or BslA) is essential protein for the maturation of *B. subtilis* biofilm. Deletion of *yuaB* presents reduced complexity of the biofilm architecture and cannot be compensated by overexpression of other extracellular components. Importantly, YuaB interact with the TasA amyloid fiber and the EPS polymer as synergistic manner to maturate biofilm. However, YuaB does not affect production of EPS and TasA [34]. In the early secretome study of *B. subtilis*, YuaB has been presumed as an extracellular protein [35]. However, recently, it has been discovered that YuaB is an cell-associated protein, which locates in the cell wall [34]. YuaB has putative signal peptide sequence, 28 amino-acid at its N-terminus, and transmembrane region. The signal sequence removed YuaB neither have wild type function nor be detected in the cytoplasm [34]. Signal peptide of YuaB is vital for secretion and function of YuaB.

In this study, YuaB was used as an anchoring motif for development of a new *B. subtilis* cell surface display system. This is the first attempt to display target protein on the *B. subtilis* cell surface by using a biofilm component, YuaB. We fused His_6 tag at the C-terminus of YuaB to analyze the possibility as anchoring motif in surface display. p43 promoter, which is overlapping promoters transcribed by *B. subtilis* σ^{55} and σ^{37} [36], is also fused at N-terminus of YuaB to stimulate production of YuaB fusion protein. We confirmed that YuaB can be used for *B. subtilis* cell surface display system by analyzing recombinant cells with flow cytometry. Furthermore, a new surface display system has been applied heavy metal adsorption and showed positive potential of its industrial utilization.

2. Materials and Methods

2.1. Bacteria and growth condition

Two protease deficient *B. subtilis* DB104 (*trpC2 nprE*

aprE) was used as a host strain [37], for the expression host of YuaB-His_6 tag. All *B. subtilis* cells were grown in Schaeffer's medium containing Difco Nutrient broth, 0.25 g $MgSO_4 \cdot 7H_2O$ and 1 g KCl per liter. After autoclaving, 1 mL of each filtered stock solution ($FeSO_4 \cdot 7H_2O$ 10^{-3} M, $MnCl_2 \cdot 4H_2O$ 10^{-2} M, and $CaCl_2 \cdot 2H_2O$ 1 M) was added and supplemented with Chloramphenicol (20 μm/mL) [38]. *E. coli* strain DH5α was used for *E. coli* transformation. All *E. coli* cells were grown under the condition at 37°C, 180rpm in LB media. Chloramphenicol (20 μm/mL) was used as a selective marker.

2.2. Construction vector and strain

pSDJH100 [38] was used for pDE100-Y1 vector construction. Chromosome of DB104 was used for PCR template. p43 promoter was amplified with primers p43-5' (5'-tca aaa**ggatcc**tgcatg cag gccggggca tat-3') and p43-3' (5'-ttg tct**ctgcag**gtgtacattcctctcttacct- 3') by PCR. pSDJH 100 and p43 PCR fragment were digested with restriction enzyme Bam I and Pst I, which is included in vector and PCR fragment, and ligated with T4 ligase. pSDJH100 containing p43 promoter was named as pDE100. *yuaB* was amplified with primers YuaB-5' (5'-**ctgcag**atgaaacgcaaattattatct-3') and YuaB-3'(5'-**ctcgag**ttaatgatgatgatgatgatg**gtcgac**gttgcaacc gcaaggctgagt-3') by PCR using DB104 chromosome as a template. His_6 tag was integrated in the YuaB-3' primer, which is indicated by shading. Vector pDE100 and PCR fragment of YuaB were digested with restriction enzyme Pst I and Xho I, then ligated with T4 ligase to construct expression vector pDE100-Y1.

2.3. Protoplast isolation

B. subtilis grew in 50 mL for 10 h and was harvested by centrifuge (10 min, 6000 rpm). Supernatant was discarded and cell pellet was washed twice with SET buffer, which contains sucrose (20% [wt/vol]), Tris-HCl (50 mM, pH 7.6), and EDTA (50 mM). After washing, pellet was suspended with 1 mL of isotonic buffer solution (TMS) containing Tris-HCl (50 mM, pH 8.0), MgCl2 (16 mM), and sucrose (33% [wt/vol]). Cell suspension was divided into 300 μL aliquots. To remove peptidoglycan layer, lysozyme (1 mg/mL final concentration) and phenylmethane sulfonyl fluoride (0.1 mM, final concentration) were added. Suspension was incubated at 37°C for 1 h. Protoplasts were pelleted (15 min, 13000 rpm) and supernatant was carefully discarded [39].

2.4. Flow cytometry analysis

DB104 and DB104(pDE100-Y1) were grown in 50 mL of Schaeffers' media for 10 h. Cells were harvested by centrifuge (10 min 6,000 rpm) and washed with phosphate buffered saline (PBS) twice. Protoplasts were washed twice with SET buffer. Bacterial cells and protoplasts were suspended in 100 mL of each PBS and SET buffer, which contains an antibody (1:220) (Rabbit Poly Anti-6-His, FITC, K0212220, Komabiotech, Korea) and reacted 1 h in the ice. After reaction, vegetative cells and protoplasts were washed 3 times with PBS and SET buffer. Analysis was taken place by Flow cytometry (Beckman Coulter Epics XL).

2.5. Heavy metal adsorption analysis

DB104 and DB104(pDE100-Y1) were grown in 100 mL of Schaeffers' media for 10 h. Cells were harvested by centrifuge (10 min, 6,000 rpm) and washed twice with distilled water. Each cell was suspended in 1 mL of sterile water. Suspended cell was measured by UV spectrometer to set the same sample O.D. Each reaction samples were fitted to O.D. 3000 (600 nm).

Copper(II) chloride solution (0.0001%) and nickel sulfate solution (0.000025 M) were used for heavy metal adsorption by bacterial cell. Each metal ion samples were prepared as standard, negative control and analyte. Final cell suspensions were inoculated in 10 mL of 0.0001% copper(II) chloride and 0.000025 M Nickel(II) sulfate. The mixtures of heavy metal and cell suspension were cultivated at 37°C, 180 rpm, 1 h for adsorption. After 1 h reaction, the mixtures were centrifuged for 10 min with 6,000 rpm to remove cell and supernatant were analyzed with atomic adsorption spectroscopy (Perkin Elmer, AAnalyst800).

3. Results

3.1. Construction of YuaB-His_6 tag expression vector and *B. subtilis* strain

In this study, a pSDJH100 vector based on pCSK1 was used, which is an *E. coli/B. subtilis* shuttle vector, for the expression of His_6 tag at the C-terminal end of YuaB anchoring motif. For the maximal vegetative surface expression of YuaB-His_6 tag on *B. subtilis* vegetative cell, we used strong p43 promoter, which is overlapping promoters transcribed by *B. subtilis* σ^{55} and σ^{37} (36), which originally transcribe cytidine/deoxycytidine deaminase in *B. subtilis*. pSDJH100 containing p43 promoter was named as pDE100.

For the promising new anchoring motif, YuaB, which is a cell-associated protein, which located in the cell wall [34], was selected. YuaB has putative signal peptide sequence, 28 amino-acid at its N-terminus, and transmembrane region, which strongly suggest it can be a good surface anchoring motif for the display of target protein.

For the efficient verification of surface expression of YuaB, it was designed that YuaB-3' (5'-**ctcgag**ttaatgatgatg atgatgatg**gtcgac**gttgcaaccgcaaggctgagt-3') primer contains

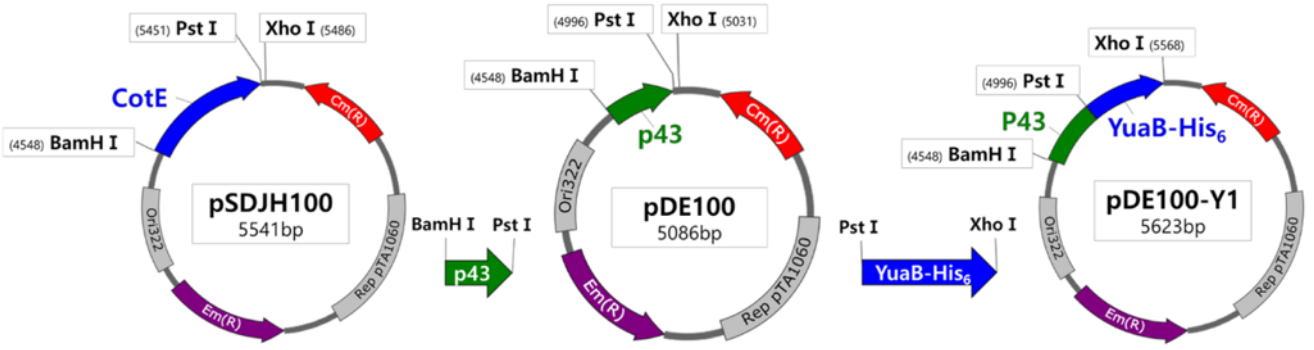

Fig. 1. Construction of pDE100 expression vector. The amplified fragment containing p43 promoter and pSDJH100 vector were digested with *Bam* HI and Pst I and ligated to yield pDE100. Constructed pDE100 and PCR fragment containing YuaB-His_6 tag were digested with Pst I and Xho I and ligated to yield pDE100-Y1.

His_6 tag at the C-terminal end of anchoring motif.

Vector pDE100 and PCR fragment of *yuaB- His_6 tag* were digested with restriction enzyme, Pst I and Xho I, and ligated with T4 ligase to construct expression vector pDE100-Y1.

The constructed expression vector was extracted using plasmid mini prep kit (plasmid SV mini, Geneall, Korea) and the correct insertion of gene into the plasmids was verified by sequencing analysis. The construction of pDE100-Y1 is shown in Fig. 1. The recombinant plasmid was transformed into strain DB104 followed by selection on LB plates containing chloramphenicol. Constructed strain was named as DB104(pDE100-Y1).

3.2. Bacterial cell growth and biofilm formation of constructed strain

The N-terminus of YuaB is located at cytoplasmic membrane of *B. subtilis*. Then it penetrates through the cell wall and, the C-terminus of YuaB is located at the extracellular region. As YuaB is a composition of cell wall, the growth curve of *B. subtilis* has been drawn to check whether the overexpressed YuaB-His_6 tag affect cell growth or cell viability. Wild type, DB104 and recombinant cell, DB104 (pDE100-Y1), was inoculated in 50 mL of Schaeffer's media with the same O.D. Both cells were grown under the condition of 37°C at 180 rpm. Every 1 h, optical density was measured. Fig. 2 shows that the overexpression of YuaB-His_6 tag does not affect cell growth and viability in planktonic growth *B. subtilis*.

YuaB plays a major role in biofilm formation. YuaB deleted mutant strain does not form biofilm. In other words, when other proteins, which involve in biofilm formation, such as DegU, TasA, lose their function, YuaB can compensate their absence [34]. pDE100-Y1 vector is designed to overexpress YuaB-His_6 tag under the control of p43 promoter. Therefore, capability of biofilm formation of DB104 and DB104(pDE100-Y1) has been compared. Both strains were inoculated into sterile flask with 50 mL of

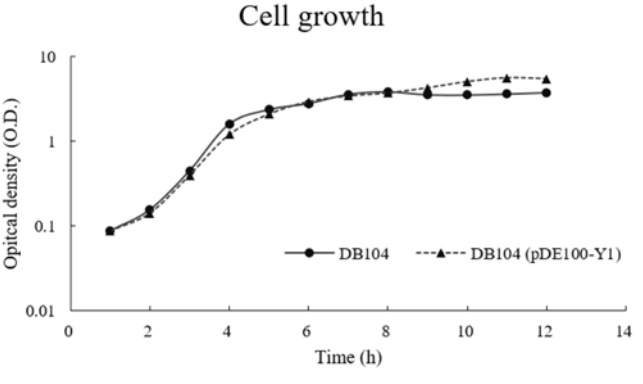

Fig. 2. Bacterial cell growth of DB104 (●) and DB104(pDE100-Y1) (▲). Both strains were grown under the condition at 37°C, 180 rpm in LB media. Chloramphenicol (20 μm/mL) was used as a selective marker for DB104(pDE100-Y1). Optical density of both cultures was measured every hour.

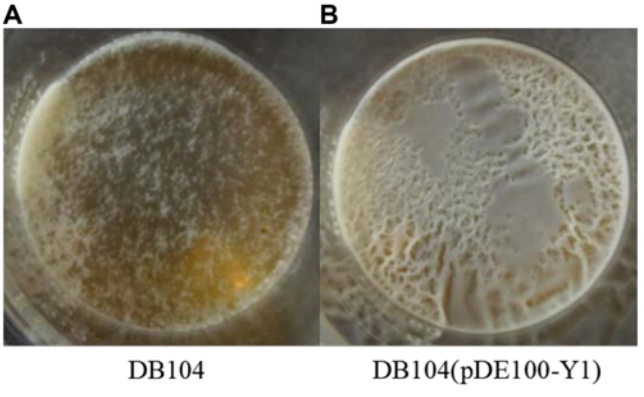

Fig. 3. Biofilm formation between wild type DB104 and DB104(pDE100-Y1), which overexpresses YuaB-His_6 tag. Both strains were inoculated into sterile flask with 50 mL of Scaeffer's media with initial same O.D. In the case of DB104(pDE100-Y1), chloramphenicol (20 μL/mL) was added in the medium. Both culture were sustained for 72 h at 30°C without shaking.

Scaeffer's media with initial same O.D. In the case of DB104(pDE100-Y1), chloramphenicol (20 μL/mL) was added in the culture medium. Both culture were sustained for 72 h at 30°C without shaking. Both strains formed biofilm at the liquid-air interface. DB104(pDE100-Y1) builds more thick and wrinkled biofilm than wild type DB104 (Fig. 3). This result indicates that DB104(pDE100-Y1) overexpressed functional p43 YuaB-His_6 tag, and this overexpressed YuaB-His_6 tag contributed the formation of wrinkled biofilm at the liquid-air interface well functions for biofilm formation even with the addition of His_6 tag.

3.3. Verification of surface display of YuaB-His_6 tag

To identify that YuaB can be used as an anchoring motif for a new *B. subtilis* cell surface display system, YuaB-His_6 tag was overexpressed in DB104. Vegetative cell of DB104 and DB104(pDE100-Y1) were both reacted with (FITC)-labeled anti-rabbit His6 for 1 h and analyzed by flow cytometry. Flow cytometric histogram of DB104(pDE100-Y1) shows increased fluorescence intensity compared that of control strain DB104 (Fig. 4A). This result demonstrates that YuaB-His_6 tag was successfully expressed and located on the *B. subtilis* cell surface. In the preceding study, YuaB was detected in three different cell fractions including the cytoplasm, membrane and non-covalently bounded membrane proteins [34]. Based on this result, we hypothesized that YuaB is located not only in the cell wall but also in the cytoplasmic membrane. To confirm the location of YuaB, protoplast, of which peptidoglycan layer is removed from vegetative cell, was prepared. YuaB-His_6 tag was observed at protoplast, DB104(pDE100-Y1)-proto (Fig. 4B). This result explains that YuaB of *B. subtilis* located at plasma membrane. Comprehensively, our study illustrates that YuaB, which is a biofilm component, can be used as an anchoring motif for a new *B. subtilis* cell surface display system.

3.4. Ni^{2+} and Cu^{2+} adsorption capacity

In this study, His_6 tag was fused at the C-terminus of YuaB for the flow cytometric analysis of surface display. Poly-

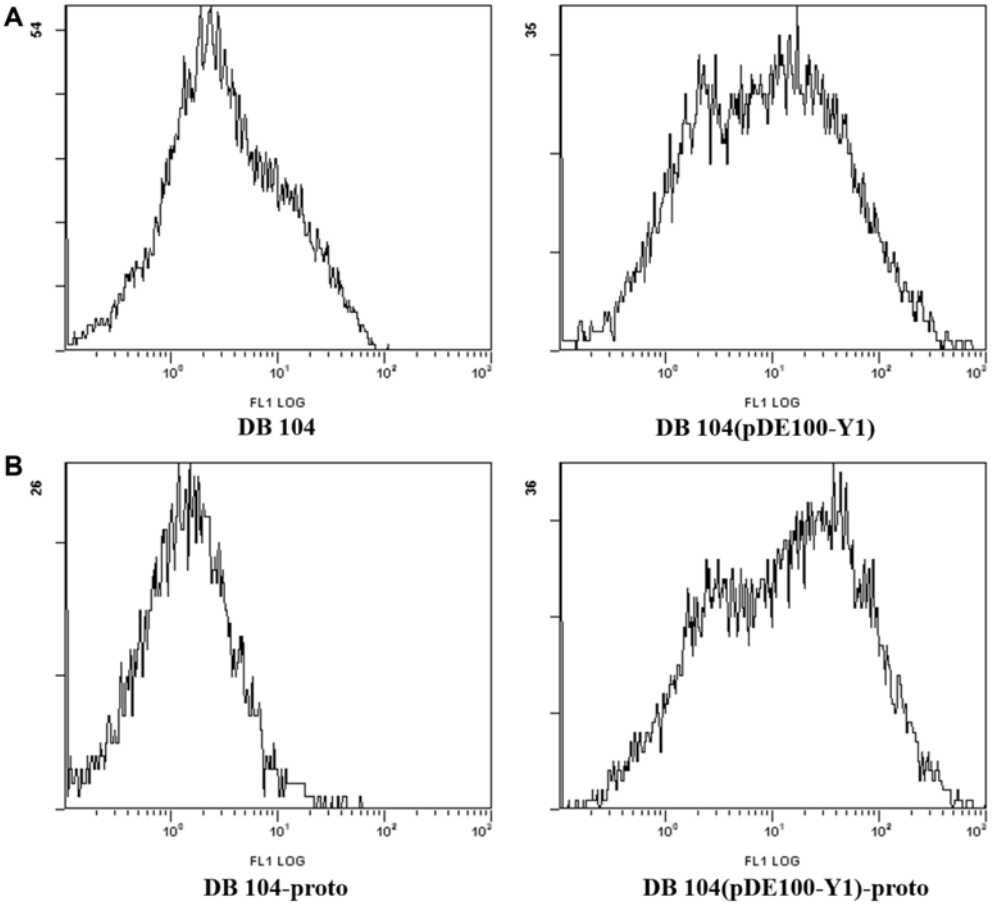

Fig. 4. Flow cytometric analysis of YuaB-His_6 tag overexpressing strain. (A) Flow cytometric histogram of DB104 (left) and DB104(pDE100-Y1) (right). DB104 is used as negative control and surface display of YuaB-His_6 tag was observed by shifted histogram of DB104(pDE100-Y1). (B) Flow cytometric histogram of protoplast analysis. DB104-proto indicates protoplast of *Bacillus subtilis* DB104. DB104(pDE100-Y1)-proto represents protoplast of DB104(pDE100-Y1).

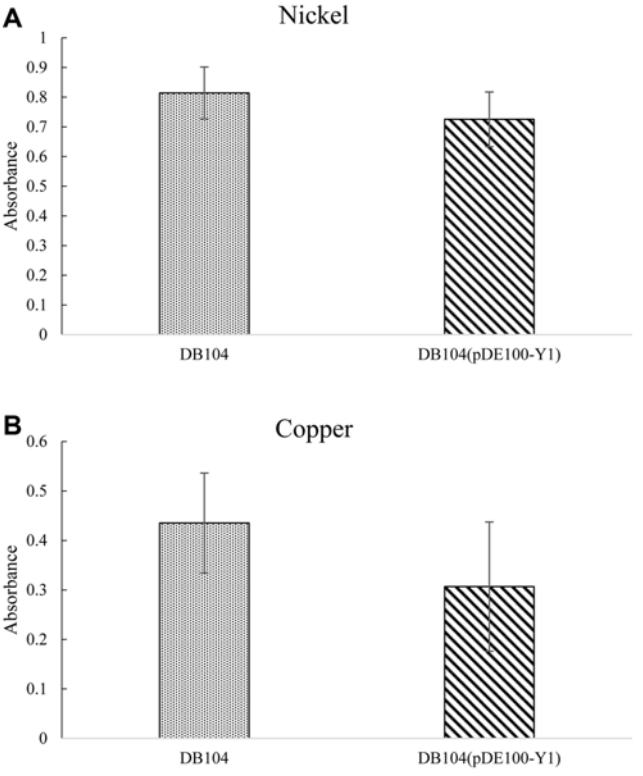

Fig. 5. Atomic adsorption analysis of heavy metal adsorption. (A) Ni^{2+} adsorption. (B) Cu^{2+} adsorption. Each graph shows the remaining amount of each metal ion in the solution after treatment with *Bacillus subtilis*. Respectively, DB104 is negative control. YuaB-His6 is recombinant DB104(pDE100-Y1), which has His6 tag at the C-terminus of YuaB. Standard means a standard control of heavy metal solutions for adsorption analysis.

histidine can adsorb divalent metal ion by forming metal-polyhistidine complex [40,41]. Accordingly, recombinant *B. subtilis*, DB104(pDE100-Y1), which has His_6 tag at C-terminus of YuaB, was utilized for heavy metal adsorption for their functionality. Cu^{2+} and Ni^{2+} were selected as the representative heavy metal for His_6 tag, which is immobilized on the *B. subtilis* cell surface using YuaB.

With standard metal solution, DB104, which is used as negative control, also went through same procedure as DB104(pDE100-Y1) to rule out technical variables. Each sample was reacted at 37°C for 1 h to optimize cell condition during the reaction. The graphs show relative remaining amount of each metal ion, Ni^{2+} and Cu^{2+}, after treatment of standard metal solution with DB104 and DB104(pDE100-Y1).

In the case of Ni^{2+} adsorption, DB104(pDE100-Y1) adsorbed 10% more Ni^{2+} of compared to DB104, which is shown as a small decrease in the relative remaining amount of metal (Fig. 5A). In the case of Cu^{2+}, DB104(pDE100-Y1) adsorbs 30% more of Cu^{2+} DB104, which is shown as a significant decrease in the relative remaining amount of metal (Fig. 5B). These data mean that YuaB-His_6 tag has different metal adsorption ability according to the nature of heavy metal ion.

YuaB served successfully for the adsorption of metal ions, Cu^{2+} and Ni^{2+}, and could be used as motif for a new *B. subtilis* cell surface display system for the other protein with various biotechnological application.

4. Discussion

B. subtilis spore display system has been well established and already applied in various biotechnological fields. Contrastively, *B. subtilis* cell surface display was hardly studied. Herein, we attempted to develop a new *B. subtilis* cell surface display system using *B. subtilis* biofilm component, YuaB. Recently, biofilm phenomena draw attention to exploit the benefits of biofilm formation [42]. Biofilm is highly structural and characteristic.

YuaB is one of the major proteins, which composes extracellular matrix of *B. subtilis* biofilm. Previously, it has been revealed that YuaB locates in the *B. subtilis* cell wall [34]. Based on this finding, YuaB has been chosen for an anchoring motif for a new *B. subtilis* cell surface display system in this study. We confirmed that YuaB was successfully overexpressed by p43 promoter and displayed on the *B. subtilis* cell surface and its surface localization is verified by flow cytometry analysis.

Moreover, from protoplast analysis, YuaB was observed in cytoplasmic membrane of *B. subtilis*. This result provides two possible hypotheses. First, YuaB locates only in the cytoplasmic membrane and penetrate through peptidoglycan layer. Second, YuaB places not only in the plasma membrane but also in the cell wall. The exact position of YuaB needs to be studied in the further studies.

Though we attached His_6 tag for the verification of surface display of YuaB, the attached His_6 tag can be used for the adsorption and removal of divalent heavy metal for the bioremediation.

Several research groups used engineered microorganisms displaying multiple histidine residues for the adsorption of heavy metal using bacterial surface display technology. We would like to focus and compare those results which were used for the adsorption of Ni^{2+} among various heavy metal adsorption researches. Several kinds of His tag, with varying length of repeating unit of His_6, have been displayed on the surface of *E. coli* cells and *B. subtilis* spore.

In the case of *E. coli*, using CS3 pili as an anchoring motif, 276.5 nmol of Ni^{2+} was removed per mg cell, which is very high value among reported cases [43].

B. subtilis spore, having higher thermos-stability and resistance to the low pH environment, lytic enzymes and

Table 1. Comparison of bioaccumulation of Ni^{2+} and Cu^{2+} with the surface displayed polyhistidine

Metal ion	Anchoring motif	Displayed peptide	Reaction condition	Bioaccumulation (nmole/mg)		Reference
				Wild type	Recombinants	
Ni^{2+}	YuaB (*Bacillus subtilis*)	His_6	37°C	4.68	5.32 (cell)	This work
	Cot B (*B. subtilis*)	His_{18}	25°C, pH7	24.79	30.66 (spore)	[44]
	Cot E (*B. subtilis*)	His_{12}	37°C, pH7	18.24	84.22 (spore)	[45]
	CS3 (*Escherichia coli*)	His_6	37°C	80 ± 2	276.5 ± 12 (cell)	[43]
Cu^{2+}	YuaB (*B. subtilis*)	His_6	37°C	11.66	15.46 (cell)	This work
	GTS1 (*Saccharomyces cerevisiae*)	His_6	37°C, pH7.8	0.11 ± 0.03	0.81 ± 0.02 (cell)	[46]

toxic chemicals, was used for similar purpose with CotB and CotE as anchoring motif. CotB-His_{18} tag displaying spore adsorbed 24% higher amount of Ni^{2+} compared to wild spore (30.66 vs. 24.79 nmol/mg spore) [44]. And, CotE-His_{12} tag displaying spore adsorbed more than 400 % higher amount of Ni^{2+} compared to wild spore (84.22 vs. 18.24 nmol/mg spore) [45].

For the adsorption of Cu^{2+}, we can find one reference using GTS1 as an anchoring motif in *S. cerevisiae* surface display system. They report elevated adsorption value (0.81 nmol/mg) compared to wild type (0.11 nmol/mg), but the absolute value of adsorption is very low due to different measurement method [46]. All these adsorption data are summarized in Table 1.

In this research, *B. subtilis* cell which expresses YuaB-His_6 tag, adsorbed relatively little amount of compared to other surface display system. It can be attributed to the difference experimental condition or measurement system between research groups. Or, it can be from the different length of binding motives such as His_6, His_{12}, and His_{18}.

Using YuaB as a new anchoring motif, *B. subtilis* cell surface display has several advantages in the industrial aspects. First, it does not need special condition for sporulation. In order to use spore, it takes time for sporulation and spore purification. Moreover, separation process after heavy metal adsorption can be easier than traditional planktonic bacterial surface display system.

Recently, two research papers focusing on *B. subtilis* cell surface display have been published. Using TasA, another important biofilm formation component, as an anchoring motif, red fluorescent protein mCherry and the antigenic peptides from *Echinococcus granulosus* parasite, Egtrp and EgA31 have been displayed on the surface of *B. subtilis* [47]. Another interesting anchoring motif was LysM cell wall binding modules. Cel8A endoglucanase is fused to LysM cell wall binding modules, and this fusion protein was expressed and bound to the surface of *B. subtilis* non-covalently [48].

Our YuaB based surface display system tends to generate biofilm easily, which means bacterial community can be easily separated from water without centrifugation or other labor-intensive procedure.

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The authors declare no conflict of interest.

Neither ethical approval nor informed consent was required for this study.

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