RESEARCH PAPER

Removal of Ni^{2+} and Cd^{2+} by Surface Display of Polyhistidine on *Bacillus subtilis* Spore Using CotE Anchor Protein

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Abstract In this paper, we report removing heavy metal using *Bacillus subtilis* spore surface display system. We used CotE protein as an anchoring motif because of its high abundance and location in the outer coat layer. And we inserted His₁₂ (double histidine 6 tag) at the C-terminal end of anchoring motif. The proper expression of CotE-His₁₂ fusion protein (22.8 kDa) was confirmed by western blot. We confirmed the surface expression of the CotE-His₁₂ fusion protein using flow cytometry. We tried Ni^{2+} and Cd^{2+} adsorption with recombinant spore DB104 (pCotE-His₁₂) and DB104 spore. The amount of adsorbed Ni^{2+} was 18.2 nmol/mg for DB104 spore and 82.4 nmol/mg for DB104 (pCotE-His₁₂) spore. In the case of Cd^{2+} , the adsorbed amount was 32.6 nmol/mg for DB104 spore and 79.1 nmol/mg for DB104 (pCotE-His₁₂) spore. This means that our spore displayed His₁₂ system can be generally applied for the removal of various kind of heavy metals in the field.

Keyword: *Bacillus subtilis*, spore surface display, CotE, metal adsorption

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1. Introduction

Pollution of heavy metals in natural ecosystems is a worldwide concern. Several metal ions, including copper, cobalt, zinc, manganese, and iron, are essential for cell growth at low concentrations. However, at high concentrations, it becomes lethal to plant and animals. Heavy metals such as cadmium, lead, nickel, and mercury are toxic to living organisms even if they are contained less than 1 mg/mL [1]. Heavy metal ions are discharged into the natural hydrosphere by a variety of industrial processes.

The most commonly used method for the removal of heavy metals is adsorption in various ways. Adsorption can be classified as physical adsorption (physisorption), chemical adsorption (chemisorption) and biological adsorption (biosorption). In physisorption, the forces of attraction between the molecules of the adsorbate and the adsorbent are the weak van der Waals' type. Since the forces of attraction are weak, the process of physisorption can be easily reversed by heating or decreasing the pressure of the adsorbate (as in the case of gases). In chemisorption, the forces of attraction between the adsorbate and the adsorbent are very strong; the molecules of adsorbate form chemical bonds with the molecules of the adsorbent present in the surface. Several chemical methods have been developed to treat and remove heavy metals from polluted ecosystems and the most important processes include precipitation-dissolution, adsorption-desorption, complexation lime coagulation [2], reverse osmosis and solvent extraction [3]. All those methods have, however, major drawbacks such as high reagent and energy requirements, or generation of toxic sludge or other waste products that require careful and expensive disposal [3]. Those disadvantages stimulated the development of biological systems to remove heavy metals from soil and aquatic ecosystems. Biosorption is a

2.3. Spore purification

Bacillus subtilis DB104 and recombinant cell were grown in Schaeffer's media for 24 h at 37°C and harvested by centrifugation (8,000 rpm, 10 min, 4°C). The pellets were washed with PBS solution and purified using Renografin (sodium diatrizoate, S4506, sigma) gradient method purified spores were confirmed under the microscope.

2.4. Western blot

Spore was suspended in SDS-page sample buffer and boiled for 8 min before SDS-page and Western blot conducted [25]. Coat protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) on a 15% gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with skim milk and detected by using anti-His6tag monoclonal antibody (H1029, Sigma) as primary antibody and anti-mouse horseradish peroxidase (HRP)-conjugated antibody (074-1806, Gaithersburg, MD) as a secondary antibody. The membranes were then washed with TBST buffer and visualized with ECL Western blotting detection reagents. The density of each band was determined with a fluorescence scanner (LAS 3000, Fuji Film).

2.5. Flow cytometry

To confirm the presence of His₁₂ of *B. subtilis* by immunofluorescence staining, purified spores were washed two times with PBS solution and then resuspended in 100 μL PBS solution containing anti-his6 tag antibody labeled FITC (1:200) (K0212220 Komabiotec, Korea) for 1 h on ice. After the spores were washed two times with PBS solution, these spores were examined under a flow cytometry (Beckman Coulter Epics XL, Germany). Spores of the host strain, *B. subtilis* DB104 (without the construction vector) was used as a control.

2.6. Ni^{2+} and Cd^{2+} adsorption measurement

Purified spores were suspended in water 1 mL and dilute

1/10. And these spores measure optical density (600 nm) by UV spectrometer and adjust the same concentration. These spores were incubated in Ni^{2+} and Cd^{2+} solution at 1 h at 37°C with 180 rpm and harvested by centrifugation (8,000 rpm, 10 min, 4°C). The supernatant was analyzed by atomic adsorption spectrometer (AAnalyst100, Perkin Elmer cooperation, USA).

3. Result

3.1. Construction of CotE-His₁₂ fusion expression vector and *Bacillus subtilis* strain

In this study, pSDJH100, which is an *Escherichia coli*/*Bacillus subtilis* shuttle vector, was used for the expression of His₁₂ at the c-terminal end of CotE anchoring motif. We designed CotE-His₁₂ reverse primers to have His₁₂ tag at the c-terminal end of anchoring motif. The amplified fragment containing CotE-His₁₂ was digested with *Bam*H I and *Sal* I and inserted into pSDJH100 digested with same restriction enzymes. 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 PCR. The construction of this vector is shown in Fig. 1. It was named pCotE-His₁₂. The recombinant plasmid was transformed into strain DB104 followed by selection on LB plates containing chloramphenicol. Constructed strain was named as DB104 (pCotE-His₁₂).

3.2. Confirmation of expression of CotE-His₁₂ fusion protein

Western blot was used to prove the proper expression of CotE-His₁₂ fusion protein. A band of approximately 22.8 kDa (equal to 20.8 kDa CotE + 2 kDa His₁₂) was detected in the extracts from pCotE-His₁₂, while no similar band in the control lane was detected. This means that which CotE-His₁₂ fusion protein is properly expressed and

Fig. 1. Construction of pCotE-His₁₂ expression vector. We designed CotE-His₁₂ reverse primers to have His₁₂ tag at the c-terminal end of anchoring motif. The amplified fragment was digested with *Bam*H I and *Sal* I and inserted into pSDJH100 digested with same enzymes.

Fig. 2. Western blot analysis carried out with purified spores of wild type strain of *B. subtilis* DB104 (lane 1) and with recombinant spores DB104 (CotE-His₁₂) (lane 2). A band around 22.8 kDa was detected in the extracts from recombinant spores, while no similar band in the control lane was detected, which is consistent with the expected molecular weight of the fusion protein CotE-His₁₂ (22.8 kDa).

located in the spore coat layer. (Fig. 2). To prove the precise localization of CotE-His₁₂ fusion protein, flow cytometry was executed.

3.3. Verification of surface display of CotE-His₁₂ fusion protein using flow cytometry

The surface localization of CotE-His₁₂ fusion protein on the spores was confirmed by flow cytometry. Purified spores of *B. subtilis* DB104 (pCotE-His₁₂) and wild type *B. subtilis* DB104 were reacted with anti-his₆ tag antibody labeled with FITC. Fluorescence intensity of the DB104 (pCotE-His₁₂) spores, expressing CotE-His₁₂ fusion protein, considerably increased in comparison with that of the control DB104 spore (Fig. 3). This result showed that CotE anchoring protein was effective to display His₁₂ tag on the outer spore surface.

3.4. Ni²⁺ and Cd²⁺ adsorption capacity

Nickel is a trace metal present in the soil, water, air and biosphere. Nickel is used for various industrial applications. However, workers in industries that deal with nickel have been reported to have a failure in the function of the lung, if it is inhaled over a long period of time. In addition, overexposure can lead to fatal diseases such as lung cancer and cirrhosis. [26]

Cadmium is brittle and ductile and has a very strong resistance to corrosion. It is mainly used in nickel-cadmium

Fig. 3. Flow cytometry of purified spores *B. subtilis* DB104 (control) and *B. subtilis* DB104 (CotE-His₁₂). DB104 spore and DB104 (CotE-His₁₂) spore was stained with FITC-labeled his₆ tag antibody. The fluorescence intensity was stronger and the histogram was shifted to the right.

battery manufacturing, pigment making, plating and so on. Even at very low concentrations, cadmium may accumulate in the kidneys, cause kidney disease, and weaken the bones when inhaled for many years. For this reasons, we selected nickel and cadmium for model heavy metal for adsorption experiments [27].

To evaluate heavy metal adsorption capability, 2.4 mg of wild type DB104 and recombinant DB104 (pCotE-His₁₂) spores were incubated in 10 mL of deionized water containing Ni²⁺ and Cd²⁺ at 37°C. Spores were then removed by centrifugation and the supernatants were analyzed for remaining Ni²⁺ and Cd²⁺ content by atomic adsorption spectroscopy. Atomic adsorption spectroscopy is a spectro-analytical procedure for the quantitative determination of chemical elements using the adsorption of optical radiation (light) by free atoms in the gaseous state. As shown in Fig. 4A, both wild type and recombinant spores were able to bind metal ions, thus reducing the amount of Ni²⁺ and Cd²⁺ in each solution. The amount of adsorbed Ni²⁺ was 18.2 nmol/mg for DB104 spore (wild type) and 82.4 nmol/mg for DB104 (pCotE-His₁₂) spore, which means that DB104 (pCotE-His₁₂) spore expressing CotE-His₁₂ were able to remove 4 times higher amount of Ni²⁺ than DB104 spore (wild type). In the case of Cd²⁺, the adsorbed amount was 32.6 nmol/mg for DB104 spore (wild type) and 79.1 nmol/mg for DB104 (pCotE-His₁₂) spore, which corresponds 2.5 times higher value. In this result, we can conclude that spore displayed His₁₂ tag increased the adsorption amount of the both metal ions.

Fig. 4. Comparison of adsorption amounts of Ni^{2+} and Cd^{2+} by the spore of DB104 and DB104 (CotE-His₁₂). (A) CotE-His₁₂ displaying spore adsorbed more than 4 times higher amount of Ni^{2+} compared to wild spore (82.42 vs. 18.24 nmol/mg spore). (B) CotE-His₁₂ displaying spore adsorbed more than 2.5 times higher amount of Cd^{2+} compared to wild spore (79.1 vs. 32.6 nmol/mg spore).

4. Discussion

In this study, we used *Bacillus subtilis* spore surface display system to solve the environmental problem by heavy metal adsorption. Several peptides of proteins have been used for metal binding such as phytochelatin [28,29] and metallothionein and multiple histidine residues [30,31]. Especially multiple histidine residues (His₆, His₁₂, His₁₈), which was widely used as antigenic motifs or protein purification, have been used as metal binding motif for their easy cloning, efficient removal of divalent metal ions. Therefore, we developed the recombinant spore of *Bacillus subtilis* expressing His₁₂ at the C-terminus of the spore coat protein CotE as heavy metal binding agent.

The use of engineered microorganisms displaying multiple histidine residues for biosorption of heavy metal has been explored by several research groups. His₆ have been expressed and displayed on the surface of *Escherichia coli* cells through fusion to the LamB [12] and cstH [32]. And polyhistidine was displayed on the surface of *E. coli* cell OmpC [33]. Gram positive bacterium *B. subtilis*, having thicker peptidoglycan cell wall was used for heavy metal removal using CS3 pili protein an anchoring motif,

B. subtilis spore, having high thermo stability and resistance in the low pH environment, lytic enzymes and toxic chemicals, was used for similar purpose with CotB as anchoring motif. Those peculiar properties make the spore potentially interesting as a bioremediation tool [1].

If we focus on heavy metal removal using *B. subtilis* spore display system, we can find only one reference, which expressed His₁₈ residues at the C-terminus of the spore coat protein CotB [1].

Though the experimental condition of each group is a little bit different, if we look at the specific value of metal adsorption by control (DB104) spore (nmol Ni^{2+} /mg spore) of both group (24.79 and 18.24), at least we can roughly compare the efficiency of each system.

CotB-His₁₈ displaying spore adsorbed 24% higher amount of Ni^{2+} compared to wild spore (30.66 vs. 24.79 nmol/mg spore). But, CotE-His₁₂ displaying spore adsorbed more than 400% higher amount of Ni^{2+} compared to wild spore (82.42 vs. 18.24 nmol/mg spore). If we compare directly, CotE-His₁₂ displaying spore adsorbed 2.68 times higher amount of Ni^{2+} than by CotE-His₁₂ displaying spore (82.42 vs. 30.66). The result is summarized in Table 1.

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

Anchoring motif (Host)	Displayed peptide	Reaction condition	Bioaccumulation (nmol/mg)		References
			Wild type	Recombinant spore	
CotB (<i>B. subtilis</i>)	His18	25°C, pH7	24.79 ^b	30.66 ^b	[1]
CotE (<i>B. subtilis</i>)	His12	37°C, pH7	18.24^a	82.42^a	This work
CS3 (<i>E. coli</i>)	His6	37°C	80 ± 2	276.5 ± 12	[32]

^aMean value is three different experiment from our result.

^bData was recalculated for correct comparison.

Table 2. Comparison of bioaccumulation of Cd²⁺ with the surface displayed polyhistidine

Anchoring motif (Host)	Displayed peptide	Reaction condition	Bioaccumulation (nmol/mg)		References
			Wild type	Recombinant spore	
CotE (<i>B. subtilis</i>)	His12	37°C, pH7	32.6^a	79.1^a	This work
LamB (<i>E. coli</i>)	His6	ND	2	7 ± 2	[12]
LamB (<i>E. coli</i>)	His12	ND	2	16 ± 2	[12]
OmpC (<i>E. coli</i>)	His6	25°C, pH5.8	10.3	18.9	[33]
OmpC (<i>E. coli</i>)	His12	25°C, pH5.8	10.3	23.9	[33]
OmpC (<i>E. coli</i>)	His18	25°C, pH5.8	10.3	26.1	[33]
OmpC (<i>E. coli</i>)	His36	25°C, pH5.8	10.3	32.0	[33]
CS3 (<i>E. coli</i>)	His6	37°C	276	656.2	[32]

^aMean value is three different experiment from our result. ND = no data

With shorter adsorbing motif (His₁₂), the possible reason for this better metal adsorption capability could be the higher amount of anchoring motif (CotE) expressed and incorporated in the spore formation process. CotE is a one of the major morphogenetic protein along with SpoIVA in spore coat assembly, which means that severe morphogenetic change is made when the protein is deleted. Its M.W. is calculated as about 20.8 kDa with 543 base pairs of ORF length [34]. CotE is synthesized early in sporulation, well before the electron-dense coat structure appears, and immuno-electron microscopy studies showed that CotE takes up a discrete subcellular location at the juncture of the inner and outer coat layers just after the formation of the sporulation septum [35]. After the forespore is engulfed to form a protoplast, the layer of CotE appears as a ring that encircles the forespore.

There was only one study which uses *E. coli* surface display system for Ni²⁺ adsorption [32]. Using CS3 (pili protein) as anchoring motif, they report 3.5 times higher value of Ni²⁺ adsorption, which is good enhancement compared to wild type. Though its absolute value of Ni²⁺ adsorption (276.5 ± 12 nmol/mg cell) is much higher than our value, it is difficult to compare with our result because of higher adsorption by host cell or spore (24.79 vs. 80 ± 2 nmol/mg cell) (Table 1).

In the case of Cd²⁺, the results of four groups were compared. Except for our experiment, the host for surface display of polyhistidine was *E. coli* altogether.

In our result, enhancement of Cd²⁺ adsorption was 2.5 times compared to wild type strain. The other groups report 4 ~ 10 times enhancement [12], 2 ~ 3 times enhancement [33], and 2.4 times enhancement [32]. Though, the precise

comparison of each display system is not easy because of wide range of adsorption amount by control strain (2 ~ 276) resulting from different host, different experimental methods (Table 2), these results implies that surface displayed polyhistidine is efficient way for the heavy metal removal.

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