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Capacity of *Bacillus thuringiensis* S-Layer Protein Displaying Polyhistidine Peptides on the Cell Surface

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Abstract

S-layer protein of *Bacillus thuringiensis* strain CTC was used as the carrier protein to display polyhistidine (poly[6His]) peptides on the cell surface. Poly(6His)$_n$ was fused with S-layer protein at two different sites, inserting just downstream of the S-layer protein homologous domain (*slh*) and replacing the non-*slh* region of S-layer protein, respectively. The two series chimeric proteins were both expressed by crystal negative *B. thuringiensis* strain 4Q7 and strain 171, respectively, as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The recombinant *B. thuringiensis* cells gained Ni^{2+}- and Cd^{2+}-binding ability and had a capacity to display up to nine copies of poly(6His). The Cd^{2+} adsorption quantity of the recombinant strain with the strongest adsorption ability was twice that of the host strain.

Index Entries: *Bacillus thuringiensis*; cell-surface display; S-layer protein; polyhistidine; *csaAB* operon; *ctc* gene.

Introduction

On the cell surface, there are many cell-surface proteins. S-layer is one of the most common surface structures on archaea and bacteria. It has been demonstrated that S-layer consists of monomolecular crystalline arrays composed of a single protein or glycoprotein. There are numerous pores identical in size and morphology on the S-layer. Most S-layers are 5–25 nm thick and cover the intact cells *(1,2)*. More than 50 S-layer genes have been cloned and sequenced so far. It has been revealed that the promoter of S-layer genes is very strong and that most S-layer proteins have an

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N-terminal secretion signal peptide that is cleaved off after translocation through the plasma membrane *(1)*. Sequence results show that the N-terminal part of many S-layer proteins possesses an S-layer-homologous domain that shows the ability to anchor on the cell surface by recognizing secondary cell-wall polymer as the binding site *(1,3)*. These features make it feasible for S-layer proteins to act as carrier protein for cell-surface display systems. There are some examples of the use of S-layer to display foreign protein on the cell surface, such as *Pseudomonas aeruginosa* pilin peptide on the cell surface of Gram-negative bacteria *Caulobacter crescentus (4)* and *Bacillus subtilis* levansucrase on the cell surface of *B. anthracis (5)*.

Theoretically, each of the cell-surface proteins has the potential ability to display foreign protein on the cell surface. To show the displaying capacity, polyhistidine peptide (poly[6His]) was usually fused to the target surface protein and then used as a marker protein. Based on the metal-binding ability of the poly(6His) peptide, it can be deduced whether the target surface protein has an ability to display a foreign protein *(6)*. For example, using poly(6His) peptide as marker, it was demonstrated that outer membrane protein C of *Escherichia coli* (OmpC) *(7)* and cell face protein A (and protein M) of *Staphylococcus xylosus* and *Staphylococcus carnosus (8)* have an ability to display foreign protein.

Although many metal-binding peptides have been expressed on the cell surface, the S-layer of *Bacillus thuringiensis* is not used as an anchoring motif to display on its cell surface. *B. thuringiensis* is a type of insect pathogenic bacterium. Recently, S-layer protein gene was isolated from this species *(9,10)*. In this study, S-layer protein of *B. thuringiensis* CTC protein *(9)* was used as carrier protein to carry out cell-surface display. This cell-surface protein shows 83% homology to that of Sap protein, the S-layer protein in *B. anthracis (11)*. To test the feasibility and capability of this surface display system, poly(6His) peptides were chosen as marker proteins. Here, we demonstrate that cell-surface layer protein of *B. thuringiensis* has an ability to display a foreign protein on its cell surface.

Materials and Methods

Bacterial Strains and Plasmids

B. thuringiensis strain CTC (serotype H_2) was isolated from soil. Plasmidless *B. thuringiensis* strain 4Q7 and strain BMB171 were provided by *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, and screened in our laboratory *(12)*, respectively. The bacterial strains and plasmids used are described in Table 1. *E. coli* and *B. thuringiensis* strains were cultivated at 37 and 28°C, respectively. Recombinant *B. thuringiensis* strains were grown in brain-heart infusion (BHI) medium when the expression and biologic function of chimeric protein was tested. Ampicillin (100 μg/mL), erythromycin (25 μg/mL), and tetracycline (20 μg/mL) were added to the medium when required.

Table 1
Bacterial Strains and Plasmids

Strains and plasmids	Characteristics	Source
B. thuringiensis		
CTC	Serotype H_2	*9*
BMB171	Serotype H3abc, plasmidless	*12*
4Q7	Serotype H_{14}, plasmidless	BGSC[a]
E. coli DH5α	*rec⁻, F⁻, endA1, gyr96, thi⁻, hsdr13(rk⁻,mk⁻), sup44, relA1*	
pBMB982	Ap^r, full length of S-layer protein gene *ctc* from strain CTC	This study
pHT304	Ap^r, Erm^r, shuttle vector	*15*
pBMB982-304	Ap^r, Erm^r, full length of *ctc* gene in pHT304	This study
pBMB622	Ap^r, Tc^r, shuttle vector, 5.7 kb	This study
pBMB-X281	Ap^r, 2.9-kb *Xba*I fragment containing upstream of gene *ctc*	*9*
pBMB-CSA	Ap^r, Tc^r, containing full length of *csaAB* operon	This study
pBMB-SHA1	Ap^r, Erm^r, $(6His)_1$ inserting in *Xba*I-*Hin*cII site of *ctc* gene	This study
pBMB-SHA2	Ap^r, Erm^r, $(6His)_2$ inserting in *Xba*I-*Hin*cII site of *ctc* gene	This study
pBMB-SHA3	Ap^r, Erm^r, $(6His)_3$ inserting in *Xba*I-*Hin*cII site of *ctc* gene	This study
pBMB-SHA6	Ap^r, Erm^r, $(6His)_6$ inserting in *Xba*I-*Hin*cII site of *ctc* gene	This study
pBMB-SHA12	Ap^r, Erm^r, $(6His)_{12}$ inserting in *Xba*I-*Hin*cII site of *ctc* gene	This study
pBMB-SHA18	Ap^r, Erm^r, $(6His)_{18}$ inserting in *Xba*I-*Hin*cII site of *ctc* gene	This study
pBMB-SHB1	Ap^r, Erm^r, $(6His)_1$ inserting in *Xba*I site of *ctc* gene	This study
pBMB-SHB2	Ap^r, Erm^r, $(6His)_2$ inserting in *Xba*I site of *ctc* gene	This study
pBMB-SHB3	Ap^r, Erm^r, $(6His)_3$ inserting in *Xba*I site of *ctc* gene	This study
pBMB-SHB9	Ap^r, Erm^r, $(6His)_9$ inserting in *Xba*I site of *ctc* gene	This study
pBMB-SHB15	Ap^r, Erm^r, $(6His)_{15}$ inserting in *Xba*I site of *ctc* gene	This study

[a]*Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH.

Plasmid pBMB622 was modified from pXI93 *(13)* by replacing the multiple cloning sites derived from pUC8 and the inserted *cry1Ab* gene with multiple cloning sites of pUC18. Finally, it contained the replication origin and tetracycline resistance gene of pBC16, pUC8 backbone, and multiple cloning sites of pUC18.

pBMB982 is made by inserting a 4.1-kb *Eco*RI-*Cla*I fragment containing a full length of S-layer protein gene *ctc* from strain CTC *(9)* into the *Eco*RI-*Acc*I site of cloning vector pIJ2925 *(14)*. After a 4.1-kb *Eco*RI-*Sph*I fragment of pBMB982 was transferred to the shuttle vector pHT304 *(15)*, pBMB982-304 resulted. The accession number of S-layer protein gene *ctc* is AJ012290.

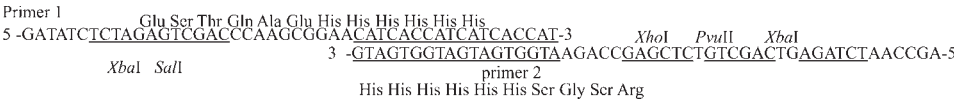

Fig. 1. Designation of poly(6His) linker by overlapping PCR.

Manipulation of DNA

DNA was manipulated as described in ref. *16*. Plasmids were transferred into *B. thuringiensis* by electroporation *(13)*.

Construction of ctc-*(6His)$_n$ Chimeric Genes*

Primers 1 and 2 were designed to amplify one poly(6His) linker by overlapping polymerase chain reaction (PCR) (Fig. 1). *Xba*I and *Sal*I sites were designed at the 5' end while *Xho*I, *Pvu*II, and *Xba*I at the 3' end of this linker. The *Xba*I-*Pvu*II fragment was used to fuse poly(6His)$_1$ with the *ctc* gene in the shuttle vector pBMB982-304 by replacing the inner *Xba*I-*Hin*cII fragment, resulting in the plasmid pBMB-SHA1, and the *Xba*I fragment was used to insert poly(6His)$_1$ into *ctc* gene at the inner *Xba*I site, resulting in pBMB-SHB1 (Fig. 2).

Based on plasmid pBMB-SHA1 and pBMB-SHB1, more copies of poly(6His) were fused with S-layer gene *ctc*. Owing to the *Sal*I- and *Xho*I-digested DNA fragment sharing the same cohesive end, the restriction sites of *Sal*I, *Xho*I, and *Eco*RI can be used to introduce one more copy of poly(6His) into *ctc* gene. Plasmid pBMB-SHA1 was digested by *Eco*RI-*Xho*I, giving an *Eco*RI-*Xho*I fragment containing *slh* domain of *ctc* gene and one copy of poly(6His). This fragment was then used to replace the *Eco*RI-*Sal*I fragment of pBMB-SHA1, resulting in plasmid pBMB-SHA2, which contained two copies of poly(6His). In the same way, *Eco*RI-*Xho*I fragment derived from plasmid pBMB-SHA1 (containing *slh* domain of *ctc* gene and one copy of poly[6His]) replaced the *Eco*RI-*Sal*I fragment of pBMB-SHA2, resulting in plasmid pBMB-SHA3, which contained three copies of poly(6His) (Fig. 3). Sets of poly(6His) were constructed in tandem to generate pBMB-SHA3, pBMB-SHA6, pBMB-SHA12, pBMB-SHA18, pBMB-SHB3, pBMB-SHB9, and pBMB-SHB15 in the same way (Fig. 3).

Construction of csaAB *Operon*

Plasmid pBMB-X281 contains a 2.9-kb *Xba*I fragment of strain CTC that consists of the 5' end of gene *ctc* encoding S-layer protein CTC and part of *csaAB* operon *(9)*. To get a full length of *csaAB* operon, *Eco*RI-*Xba*I fragment containing *csaAB* operon upstream was amplified by performing PCR with Primer 3 (AGCTGAATTCAGCATGTTGTAAAC) and Primer 4 (CCACTATATATTATTGTAATAATTCTAGA) from genomic DNA of strain CTC. This 1.7-kb *Eco*RI-*Xba*I fragment inserting into shuttle vector pBMB622 resulted in pBMB-CSAT. Then an *Xba*I-*Hin*dIII fragment of

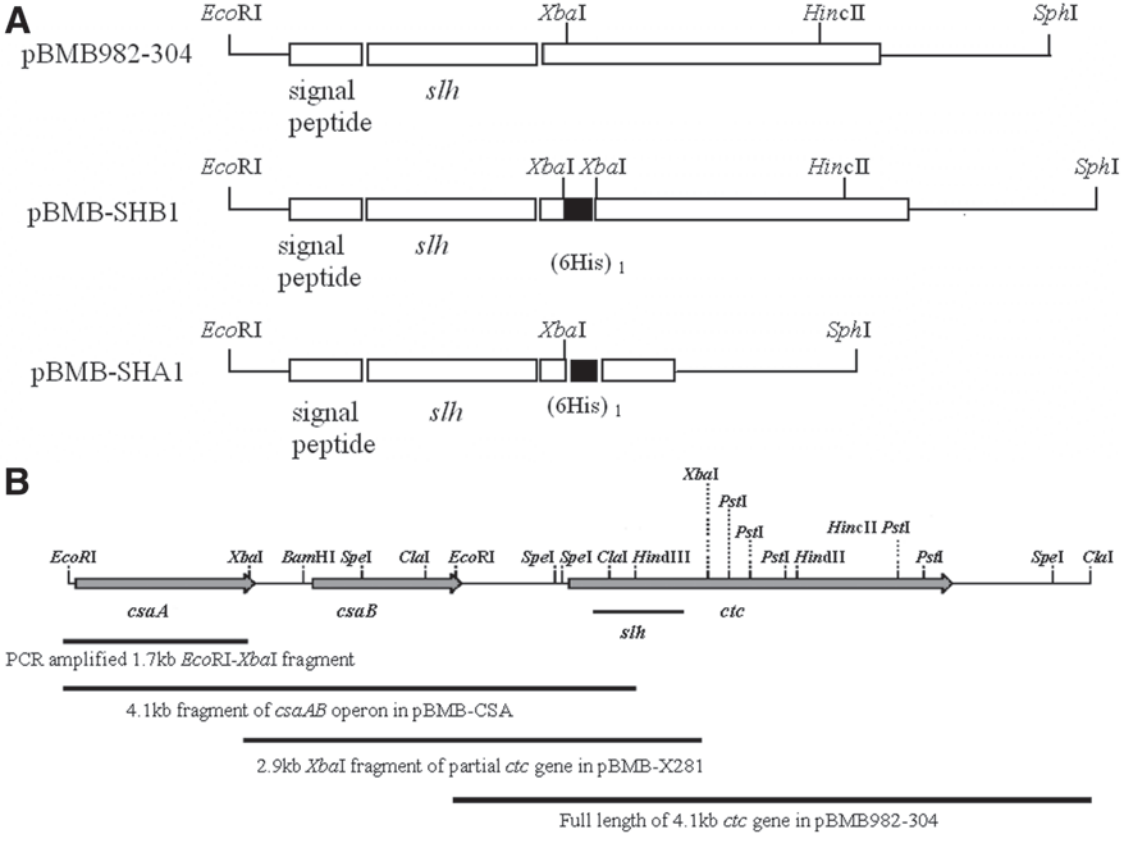

Fig. 2. **(A)** S-layer protein gene *ctc* and fusion gene *ctc*-$(6His)_1$, encoding one copy poly (6Histidine), was inserted into *Xba*I site or replaced the *Xba*I-*Hinc*II fragment of pBMB982-304, pBMBSHB1, and pBMBSHA1 was constructed respectively. The dark rectangles represent $(6His)_1$, *slh* means S-layer protein homologous domain. **(B)** Gene map of S-layer protein gene *ctc* and its upstream operon *csaAB* (7.1 kb) and location of the inserts in some recombinant plasmids.

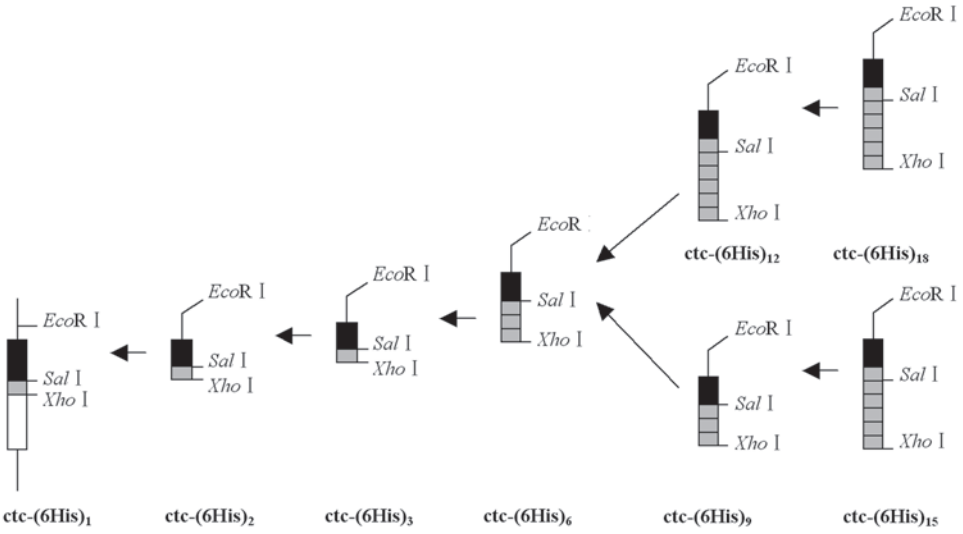

Fig. 3. Construction of ctc-$(6His)_n$ fusion genes. Black rectangles represent the *slh* anchoring motif, gray ones represent 6His clusters, and the white one is a structural segment of gene *ctc*.

pBMB-X281 contained downstream of *csaAB* operon was inserted into pBMB-CSAT, resulting in pBMB-CSA (Fig. 2B).

Cell Adhesion to Ni–Ni^{2+}-Nitilotriacetic Acid–Agarose Beads

Ni^{2+}-nitilotriacetic acid (NTA)–agarose beads (Qiagen) were used to examine the Ni^{2+} adhesion ability of recombinant strains. Bacterial strains were cultivated in BHI medium overnight, and cells were washed three times with 0.85% (w/v) NaCl, then suspended in the same concentration of NaCl. The suspension was mixed with Ni-NTA-agarose beads and propidium iodide was used to stain the mixture *(6,7)*. Under fluorescence microscope the samples were examined.

Adsorption Ability of Cd^{2+}

Cell adsorption for Cd^{2+} was measured by atomic absorption. Strains being tested were cultivated in BHI at 28°C overnight. Bacteria cells were pelleted, washed three times with 0.85% (w/v) NaCl, and suspended again. The concentration of cells in the suspension was controlled at 2×10^9/mL. Suspension (0.5 mL) and an equal volume of $CdCl_2$ (50 mg/mL in 0.85% [w/v] NaCl, pH 5.8) was mixed and then incubated for 24 h with shaking at 28°C. The cells were pelleted again, washed twice with 0.85% (w/v) NaCl, and treated overnight with 70% nitric acid *(6,7)*. This acid treatment was diluted and measured at a wavelength of 228.8 nm with a spectrophotometer (ICP-OES; Varian).

Results

Expression of CTC-(6His)$_n$ Fusion Proteins

Because S-layer CTC protein shows 83% homology to S-layer protein in *B. anthracis*, in which *csaAB* operon is required for S-layer anchoring *(3)*, and in order to make sure that S-layer protein anchored on the cell surface efficiently, *csaAB* operon was built in a host strain to display foreign proteins.

A series of recombinant BMB171 (pBMB-CSA, pBMBSHAn) and 4Q7 (pBMB-CSA, pBMBSHBn) was constructed to determine whether S-layer protein can be used to direct poly(6His)$_n$ to express on the cell surface of *B. thuringiensis*. When cells were grown to logarithmic phase, cell-surface proteins were prepared as described by Mesnage et al. *(5)* and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

For the BMB171 (pBMB-CSA, pBMBSHAn) series, the fusion proteins were expressed and the molecular weight of these proteins was increased in proportion to the copies of poly(6His) (Fig. 4A). The predicted size of the fusion proteins is 48.2, 49.6, 51.0, 55.2, 63.6, and 72.0 kDa when *n* is 1, 2, 3, 6, 12, and 18, respectively. Figure 4 shows that when *n* ranged from 1 to 6, the fusion protein expressed clearly. Because of the heavy bands of background proteins at 63.6 and 72.0 kDa, the predicted fusion proteins may have been blocked by the background proteins or did not exist when *n* was 12 and 18.

For the 4Q7 (pBMB-CSA, pBMBSHBn) series, the fusion proteins were expressed similar to that of the BMB171 (pBMB-CSA, pBMBSHAn) series. The predicted size for those fusion proteins is 101.8, 103.2, 104.6, 113.0, and 121.4 kDa when *n* is 1, 2, 3, 9, and 15, respectively. The apparent molecular mass of CTC-(6His)$_n$ increased when *n* was from 1 to 9 (Fig. 4B). As shown in Fig. 4, the predicted fusion protein CTC-(6His)$_{15}$ did not exist.

In addition, Fig. 4 shows that the expression of fusion proteins was poor compared to the background proteins. S-layer protein can be highly expressed in strain CTC, but the expression level decreased strongly in strain BMB171 and strain 4Q7 (results not shown). We do not know whether there are any factors that inhibit its expression or whether the proteins are degraded in the nonmother cell.

Binding Capacity of (6His)$_n$ Fusion Protein to Ni^{2+} Ion

NTA-agarose beads were used to examine the Ni^{2+} adhesion ability of recombinant strains. A fluorescence microscope was used to make images for the binding. Cells stained with propidium iodide sent out fluorescence. If recombinant cells bind to agarose beads, a visible outline of beads can be seen under a fluorescence microscope. Figure 5 shows that there was no visible fluorescent outline of beads for host BMB171 (pBMB-CSA) and 4Q7 (pBMB-CSA). This means that they did not bind to the agarose beads. BMB171 (pBMB-CSA, pBMB-SHAn) and 4Q7 (pBMB-CSA, pBMB-SHBn)

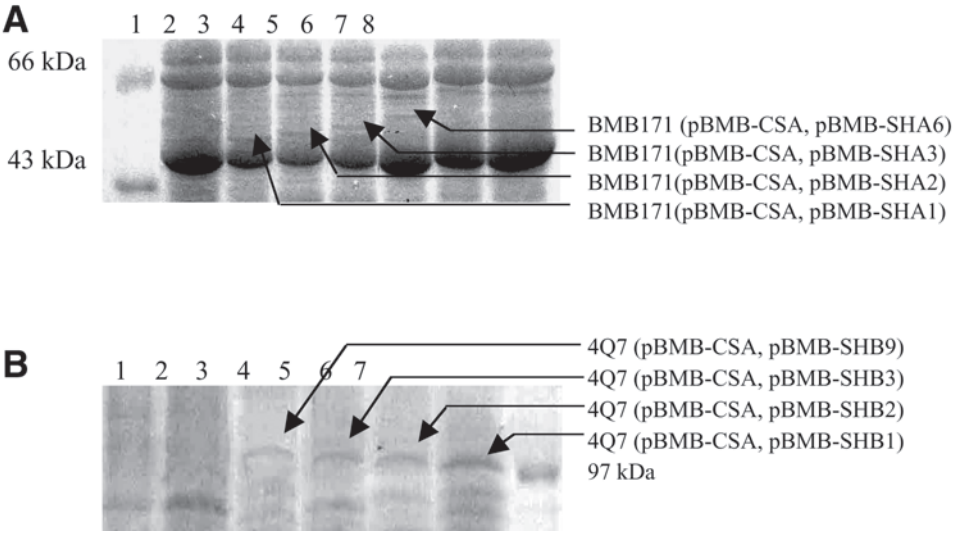

Fig. 4. SDS-PAGE of recombinant strains BMB171 (pBMB-CSA, pBMB-SHAn) and 4Q7 (pBMB-CSA, pBMB-SHBn). **(A)** Lane 1, molecular weight marker; lane 2, BMB171 (pBMB-CSA); lane 3, BMB171 (pBMB-CSA, pBMB-SHA1); lane 4, BMB171 (pBMB-CSA, pBMB-SHA2); lane 5, BMB171 (pBMB-CSA, pBMB-SHA3); lane 6, BMB171 (pBMB-CSA, pBMB-SHA6); lane 7, BMB171 (pBMB-CSA, pBMB-SHA12); lane 8, BMB171 (pBMB-CSA, pBMB-SHA18). **(B)** Lane 1, 4Q7 (pBMB-CSA); lane 2, 4Q7 (pBMB-CSA, pBMB-SHB15); lane 3, 4Q7 (pBMB-CSA, pBMB-SHB9); lane 4, 4Q7 (pBMB-CSA, pBMB-SHB3); lane 5, 4Q7 (pBMB-CSA, pBMB-SHB2); lane 6, 4Q7 (pBMB-CSA, pBMB-SHB1); lane 7, molecular weight marker.

were able to bind to the beads because a clear outline of the beads was visible under the microscope (Fig. 5). For both of the fusion types, when *n* ranged from 1 to 9, fusion proteins bound to Ni^{2+} ion. However, the binding ability of BMB171 (pBMB-CSA, pBMB-SHA12) was weak, and 4Q7 (pBMB-CSA, pBMB-SHB15) did not show binding activity. This indicated that the chimeric protein CTC-$(6His)_n$ was expressed on the surface of the recombinant cells and displayed active biologic function.

Cd^{2+} Adsorption Ability of Recombinant Strains

In Fig. 5 it is difficult to distinguish the metal adsorption capacity of these recombinant cells accurately. To determine how much stronger the metal bioaccumulation capacity of recombinant strains is over that of host strains, atomic absorption was used to measure the adsorption of Cd^{2+} ion for all recombinant strains. Strains being tested were cultivated in BHI at 28°C overnight. Samples were prepared and measured at a wavelength of 228.8 nm. At this wavelength, the Cd^{2+} concentration of three standard samples was measured, and the correlation reached 99.9999%. As shown in Fig. 6, the strains producing poly$(6His)_n$ fusion protein accumulated a substantially higher amount of Cd^{2+} ion than the cells not expressing the protein. BMB171 (pBMB-CSA) could adsorb 1.42×10^7 Cd^{2+} ions/cell, while

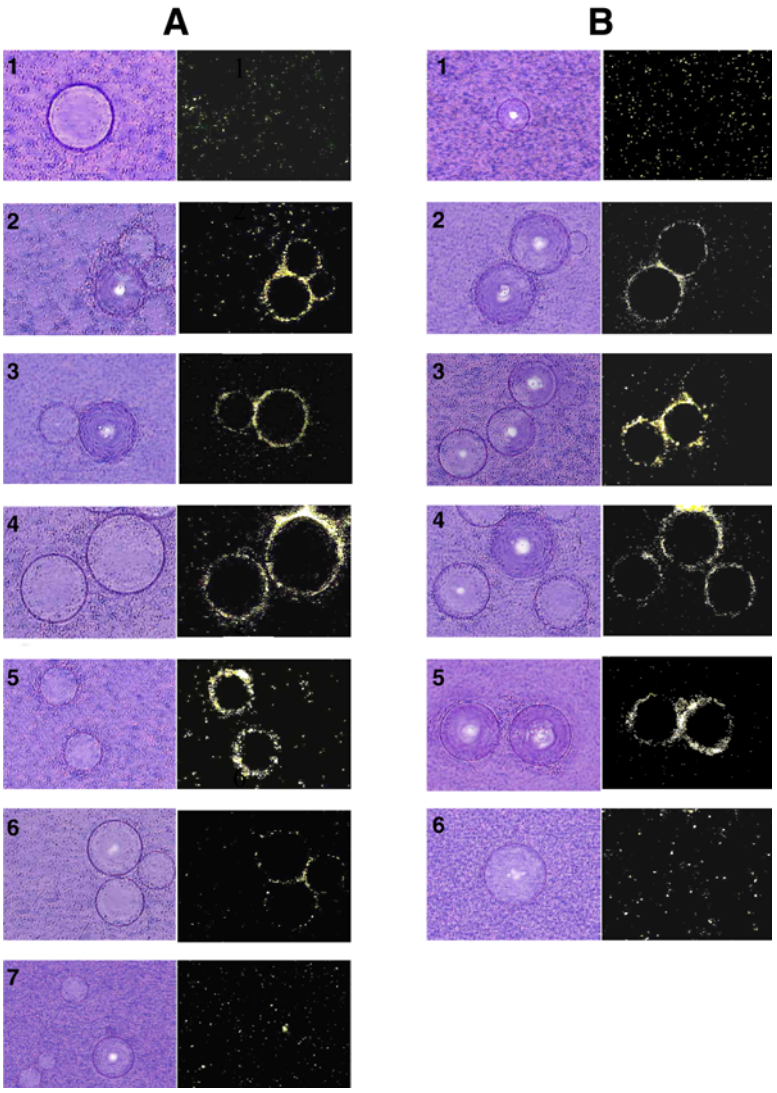

Fig. 5. Adhesion of recombinant strains to Ni-NTA-agarose beads. Optical micrographs (**left**) and matching fluorescent micrographs (**right**) are shown. **(A)** 1, BMB171 (pBMB-CSA); 2, BMB171 (pBMB-CSA, pBMB-SHA1); 3, BMB171 (pBMB-CSA, pBMB-SHA2); 4, BMB171 (pBMB-CSA, pBMB-SHA3); 5, BMB171 (pBMB-CSA, pBMB-SHA6); 6, BMB171 (pBMB-CSA, pBMB-SHA12); 7, BMB171 (pBMB-CSA, pBMB-SHA18). **(B)** 1, 4Q7 (pBMB-CSA); 2, 4Q7 (pBMB-CSA, pBMB-SHB1); 3, 4Q7 (pBMB-CSA, pBMB-SHB2); 4, 4Q7 (pBMB-CSA, pBMB-SHB3); 5, 4Q7 (pBMB-CSA, pBMB-SHB9); 6, 4Q7 (pBMB-CSA, pBMB-SHB15).

the adsorption capacity of BMB171 (pBMB-CSA, pBMB-SHAn) was 3.12×10^7/cell, 3.28×10^7/cell, 3.39×10^7/cell, 4.00×10^7/cell, 2.73×10^7/cell, and 1.44×10^7/cell when n was 1, 2, 3, 6, 12, and 18, respectively. As for the 4Q7 (pBMB-CSA, pBMB-SHBn) series, the absorption ability was 1.38×10^7/cell, 2.81×10^7/cell, 3.03×10^7/cell, 3.11×10^7/cell, 3.74×10^7/cell, and $1.43 \times$

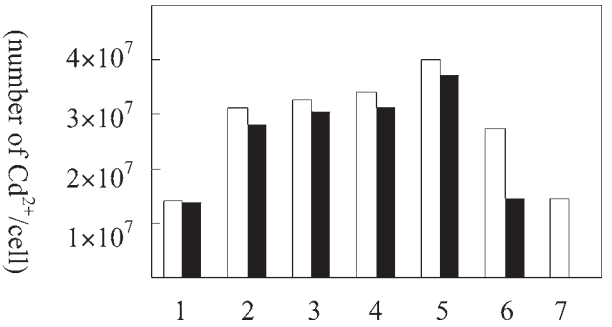

Fig. 6. Adsorption of Cd^{2+} by recombinant strains BMB171 (pBMB-CSA, pBMB-SHAn) and 4Q7 (pBMB-CSA, pBMB-SHBn). White rectangles represent the adsorption quantity of BMB171 (pBMB-CSA, pBMB-SHAn) and black ones represent that of 4Q7 (pBMB-CSA, pBMB-SHBn). 1, BMB171 (pBMB-CSA), 4Q7 (pBMB-CSA); 2, BMB171 (pBMB-CSA, pBMB-SHA1), 4Q7 (pBMB-CSA, pBMB-SHB1); 3, BMB171 (pBMB-CSA, pBMB-SHA2), 4Q7 (pBMB-CSA, pBMB-SHB2); 4, BMB171 (pBMB-CSA, pBMB-SHA3), 4Q7 (pBMB-CSA, pBMB-SHB3); 5, BMB171 (pBMB-CSA, pBMB-SHA6), 4Q7 (pBMB-CSA, pBMB-SHB9); 6, BMB171 (pBMB-CSA, pBMB-SHA12), 4Q7 (pBMB-CSA, pBMB-SHB15); 7, BMB171 (pBMB-CSA, pBMB-SHA18).

10^7/cell when *n* was 0, 1, 2, 3, 9, and 15, respectively. The adsorption ability of Cd^{2+} increased as the number of poly(6His) units increased. The greatest Cd^{2+} adsorption capacity of recombinant cells was twice that of the host strain. This confirmed that 6His clusters were displayed on the surface of these recombinant cells that took advantage of the signal peptide and *slh*-anchoring motif of the S-layer protein CTC.

Discussion

S-layer protein from *B. thuringiensis* strain CTC was demonstrated to have an ability to display poly(6His)$_n$ peptides on the cell surface of *B. thuringiensis*, and this displaying capacity of S-layer protein was tested. We showed that *B. thuringiensis* S-layer protein successfully directed the expression of poly(6His) in two ways. One was by connecting target protein to the N-terminal *slh* part of the S-layer protein. The other was by sandwiching the target protein downstream of the *slh* domain in the full length of S-layer protein. In other reports, OmpC protein displaying poly(6His) was achieved the second way *(7)*, and *B. anthracis* S-layer displaying levansucrase and tetanus toxin *(5,17)* was achieved the first way.

When displaying poly(6His)$_n$ peptides, the efficiency was good when 1 to 9 copies of poly(6His) peptides were displayed, whereas that of more than 12 copies was less or none. This may be because enzyme digestion easily attacks the peptides with too many poly(6His) copies. *B. thuringiensis* is a soil bacterium that inhabits many environments. It is well known that some *B. thuringiensis* isolates are toxic to insects. Biopesticides and transgenic plants developed from *B. thuringiensis* or its insecticidal genes were shown to be safe to humans and the environment *(18)*. We demon-

strated that displaying poly(6His) clusters enhanced the metal adsorption ability of recombinant strains. If the expression system is improved, *B. thuringiensis* S-layer could be used as bioadsorbents, recombinant vaccines, and living-cell enzymes.

Acknowledgments

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