*J. Microbiol. Biotechnol.* (2009), **19**(5), 495–501
doi: 10.4014/jmb.0802.163
First published online 30 January 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Spore Display Using *Bacillus thuringiensis* Exosporium Protein InhA

**Park, Tae Jung[1], Soo-Keun Choi[2], Heung-Chae Jung[2], Sang Yup Lee[1,3], and Jae-Gu Pan[2,4]***

[1]*Department of Chemical and Biomolecular Engineering (BK21 Program), BioProcess Engineering Research Center, Center for Systems and Synthetic Biotechnology, and Institute for the BioCentury, KAIST, Daejeon 305-701, Korea*
[2]*National Laboratory for Microbial Display, GenoFocus, Daejeon 305-811, Korea*
[3]*Department of Bio and Brain Engineering, and Bioinformatics Research Center, KAIST, Daejeon 305-701, Korea*
[4]*Systems Microbiology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea*

Received: February 28, 2008 / Revised: July 30, 2008 / Accepted: August 13, 2008

**A new spore display method is presented that enables recombinant proteins to be displayed on the surface of *Bacillus* spores *via* fusion with InhA, an exosporium component of *Bacillus thuringiensis*. The green fluorescent protein and β-galactosidase as model proteins were fused to the C-terminal region of InhA, respectively. The surface expression of the proteins on the spores was confirmed by flow cytometry, confocal laser scanning microscopy, measurement of the enzyme activity, and an immunogold electron microscopy analysis. InhA-mediated anchoring of foreign proteins in the exosporium of *Bacillus* spores can provide a new method of microbial display, thereby broadening the potential for novel applications of microbial display.**

**Keywords:** *Bacillus thuringiensis*, spore display, exosporium, InhA

The *Bacillus cereus* group, which includes *B. anthracis* and *B. thuringiensis*, is characterized by the presence of an exosporium, a loose-fitting outermost layer surrounding the spore's outer coat [8, 24, 27]. The particular adherence and hydrophobic property conferred by the exosporium, which forms the initial host–pathogen contact, suggest that the exosporium may be significant to spore pathogenicity [27]. Several genes encoding exosporium proteins have already been identified from the *B. cereus* group [3], including *inhA* and *inhB* in *B. thuringiensis* [3, 7], a series of *exs* genes in *B. cereus* [28], and the *bclA* gene in *B. anthracis* [26, 27].

Numerous researchers have also developed cell-surface engineering techniques for the display of heterologous proteins. Many of these strategies involve the fusion of target genes or partner genes encoding surface-anchoring motifs or outer-membrane proteins, followed by the expression of the heterologous protein in the relevant host cell. Experiments utilizing such cell-surface display systems have proven to be powerful tools for investigating important biomolecular interactions, including protein–protein, antigen–antibody, and enzyme reactions [6, 18]. In particular, microbial cell-surface display systems have shown great promise for the development of live-cell patterning [23], the high-throughput screening of peptide and antibody libraries [2, 4], live vaccine development [17, 19], bioconversion using whole-cell biocatalysts [11, 15], and bioadsorption [29]. In recent years, some researchers have focused on developing spore surface display techniques with *B. subtilis* spores, which have the advantages of being highly resistant to destructive environmental stimuli and having the ability to remain dormant for extended periods of time [9, 14, 19–21], in part due to the characteristics of the coat proteins that form the outer layers of the spores [3, 7]. The previously developed spore-display systems in *B. subtilis* have involved the fusion of target proteins to the outer-coat proteins, such as CotB, CotC, and CotG [8, 10, 13, 14, 19]. However, since the absence of any one coat protein or its modification by fusion with another protein can lead to incorrect assembly and structural alterations due to the coat protein network [12], it is anticipated that fusion with a loosely attached exosporium protein will not affect the structure and function of the spores. Yet, no previous study has examined the use of exosporium proteins as a fusion partner for spore display. Accordingly, this study reports on a novel system that enables recombinant proteins to be displayed on the surface of *B. thuringiensis* spores *via* C-terminal fusion to InhA, an exosporium protein that is expressed at the onset of sporulation [8, 14, 23, 28].

*Corresponding author
Phone: +82-42-860-4483; Fax: +82-42-860-4488;
E-mail: jgpan@kribb.re.kr

## Materials and Methods

### Bacterial Strains and Plasmids

All the bacterial strains and plasmids used in this study are listed in Table 1. All the polymerase chain reaction (PCR) experiments were performed using a PCR Thermal Cycler T1 (BioMetra, Germany) with a High Fidelity PCR System. The restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA, U.S.A.). The DNA sequences of all the clones were confirmed using an automatic DNA sequencer (ABI Prism Model 377; Perkin Elmer, Waltham, MA, U.S.A.). The other general molecular biological experiments were all carried out following standard procedures [25].

The *inhA* gene containing its promoter was amplified by a PCR from the genomic DNA of *B. thuringiensis* subspecies *kurstaki* HD1 using the following two primers: 5'-ATAAGAATGCGGCCGCATG-TAATTCCTCCCTAATTAT-3' and 5'-GCTCTAGAACGATATAAA-CGAAC-3'. The amplified fragments were then digested with the restriction enzymes NotI and XbaI (sites underlined) and cloned into the *Bacillus* plasmid pS to make pSD1. The DNA fragments encoding the enhanced green fluorescent protein (EGFP) and β-galactosidase (β-Gal) were then obtained by PCR amplification from the plasmids pEGFP (Clontech, Mountain View, CA, U.S.A.) and pDG1728 (*Bacillus* Genomic Stock Center, Columbus, OH, U.S.A.) as templates, respectively.

For the cloning of the *egfp* gene, an XbaI restriction site was first introduced at the 5' end of the oligonucleotide (5'-GCTCTAGAAT-GCACGGCAAAACCCAGGCGACCAGCGGCACCATCCAGAGC ATGCATGGTAAAACGCAAGCCACGTCTGGTACGATT-3'), which was then used as the first forward primer for the primary PCR. Meanwhile, an EcoRI restriction site and stop codon were introduced at the 5' end of the oligonucleotide (5'-GGAATTCTTACTTGTACA-GCTCGTCCAT-3'), which was then used as the reverse primer. The primary PCR was carried out with the first forward primer and reverse primer. To obtain the *egfp* gene product, a second PCR was carried out with the second forward primer and reverse primer. The *egfp* PCR product including the XbaI and EcoRI restriction sites was then ligated into the same site in pSD1, yielding pSD-EGFP.

For the cloning of the *lacZ* gene, an XbaI restriction site was introduced at the 5' end of the oligonucleotide (5'-GCTCTAGAGT-GGAAGTTACTGACGTAAGAT-3'), which was then used as the forward primer. Meanwhile, an EcoRI restriction site and stop codon were introduced at the 5' end of the oligonucleotide (5'-GGAATTC-TCGAGTTATTTTTGACACCAGACCAA-3'), which was then used as the reverse primer. Thereafter, the *lacZ* PCR product including the XbaI and EcoRI restriction sites was ligated into the same site in pSD1, yielding pSD-LacZ.

The cloned plasmid was transformed into *B. thuringiensis* 4Q7 by electroporation, which was performed using a Gene Pulser (Bio-Rad, Hercules, CA, U.S.A.) with a 0.2-cm cuvette. The voltage was adjusted to 2.5 kV, the capacitance to 25 μF, and the resistance was 400 Ohms.

### Purification of Spores

*Bacillus* strains displaying β-Gal were cultivated in DSM sporulation media [21] at 30°C and 250 rpm for 48–60 h. Spores mixed with vegetative cells were then harvested from 50 ml of the culture by centrifugation (10,000 ×*g*, 10 min) and resuspended in 200 μl of 20% (wt/vol) urografin (Sigma, St. Louis, MO, U.S.A.). To collect just the free spores, the suspension was gently layered over 1 ml of 50% (wt/vol) urografin in a 1.5-ml microcentrifuge tube, and then centrifuged for 10 min at 4°C and 16,000 ×*g*. The collected pellets containing just free spores were stored at −4°C after washing and resuspension at ~ $1.0\times10^8$ CFU/ml in a phosphate-buffered saline (PBS) solution.

### Flow Cytometry Analysis

The β-Gal-displaying spores were incubated with mouse anti-β-Gal monoclonal antibodies (Sigma) at 30°C for 1 h, and then washed three times with PBS to remove any unbound reagents. Next, the spores were incubated with a rabbit anti-mouse IgG-FITC conjugate (Sigma) as a secondary antibody at 30°C for 1 h, and washed three times with PBS. The spores were then collected and resuspended in PBS, and their binding affinities examined using a FACSCalibur flow cytometer and the Cell Quest Pro software (BD Bioscience, San Jose, CA, U.S.A.). The spores displaying EGFP and β-Gal were

**Table 1.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant genotypes | Reference or source |
|---|---|---|
| Strains | | |
| *Escherichia coli* JM109 | *F'traD36 proA+B+ lacIq Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17* | ATCC[a] 53323 |
| *B. thuringiensis* subsp. *israelensis* | 4Q7, plasmidless mutant of *B. thuringiensis* var. *israelensis* harboring pTX14-1, pTX14-2, and pTX14-3 | BGSC[b] 4Q7 |
| *B. thuringiensis* subsp. *kurstaki* | HD1, wild-type isolate | BGSC 4D1 |
| Plasmids | | |
| pEGFP | *egfp*, *bla* | Clontech[c] |
| pDG1728 | *bla, erm, spc, spoVG-lacZ* | BGSC |
| pS | *cat* | This study |
| pSD1 | *cat*, *inhA* | This study |
| pSD-EGFP | *cat*, *inhA*, *egfp* | This study |
| pSD-LacZ | *cat*, *inhA*, *lacZ* | This study |

[a]American Type Culture Collection, Manassas, VA, U.S.A.
[b]*Bacillus* Genetic Stock Center, Columbus, OH, U.S.A.
[c]Clontech, Mountain View, CA, U.S.A.

analyzed for their relative fluorescence intensity using an FL1 green fluorescence detector with a 530/30 nm bandpass filter, respectively. The mean value (M) indicates the mean fluorescence intensity obtained by the FL1 detectors.

**Fluorescence Microscopy**

The purified spores displaying EGFP were harvested by centrifugation at 10,000 ×*g* for 1 min at room temperature, washed, and resuspended at ~ $1.0\times10^8$ CFU/ml in PBS. The EGFP-displaying spores were mounted on poly-L-lysine-coated glass slides (Cel & Associates, Pearland, TX, U.S.A.) and analyzed under an LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany). The samples were excited at 488 nm with an argon laser, and the images filtered with a long-pass 505 nm filter. All the final images were generated from 3–5 serial images made by automatic optical sectioning.

**β-Galactosidase Activity**

The purified β-Gal-displaying spores (~ $1.0\times10^8$ CFU/ml) and free-formed β-Gal (20 unit/ml) were combined to start the reaction. The reaction mixture also contained 3 μl of $MgCl_2$ (1 mM) and β-mercaptoethanol (45 mM), plus 267 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG, 3 mM) dissolved in a 0.1 M sodium phosphate buffer (pH 7.5). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 500 μl of 1 M $Na_2CO_3$, and the absorption of the liberated *o*-nitrophenol measured at 420 nm. One unit of β-Gal was defined as the amount of enzyme that hydrolyzed 1 μmole of ONPG to *o*-nitrophenol and D-galactose per minute per spore density ($A_{600}$) at 37°C.

**Protease Accessibility**

The β-Gal-displaying spores were purified and washed with a PBS buffer. The spores were then resuspended in PBS containing proteinase K, bacterial subtilisin, and pancreatic trypsin (Sigma) at 0.2 mg/ml, respectively. After incubation at 37°C for 30 min, the spores (~ $1.0\times10^8$ CFU/ml) were washed three times and resuspended in PBS. The residual enzyme activity of the remaining β-Gal-displaying spores was measured and a flow cytometry analysis performed as described above.

**Transmission Electron Microscopy (TEM)**

The spore samples displaying β-Gal were incubated with mouse anti-β-Gal monoclonal antibodies (Sigma) in PBS at 30°C for 1 h and washed three times with PBS. The spores were then incubated with a goat anti-mouse immunoglobulin G (IgG)-gold conjugate (Sigma) as a secondary antibody at 30°C for 1 h and washed three times with distilled water. Thereafter, the samples were placed on carbon and Formvar-coated grids (Electron Microscopy Sciences, Hatfield, PA, U.S.A.) and examined after freeze drying using a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands) with an accelerating voltage of 80 kV.

## RESULTS AND DISCUSSION

### Spore Display of Green Fluorescent Protein and β-Galactosidase

To test the InhA-mediated spore surface display, two model proteins, the monomeric EGFP and tetrameric β-Gal, were used. The detection of fluorescence was then used to monitor the expression level of EGFP fusion on the spore surface [13]. Since the surface display of active tetrameric β-Gal is generally regarded as impossible, as its monomer subunit has to be initially secreted from the cell and then assembled outside the cell, another objective of this study was to confirm the direct surface-display of active β-Gal.

To examine the targeting of the EGFP on the spore surface, the EGFP gene was fused to the C-terminal of *inhA* of *B. thuringiensis*, as described in the Materials and Methods section. EGFP-displaying *B. thuringiensis* spores were then prepared through urografin gradient centrifugation and analyzed by flow cytometry (Fig. 1A) and confocal microscopy (Fig. 1B). In the flow cytometry analysis, the fluorescence intensity of the purified EGFD-displaying spores that carried an InhA fusion protein was higher and

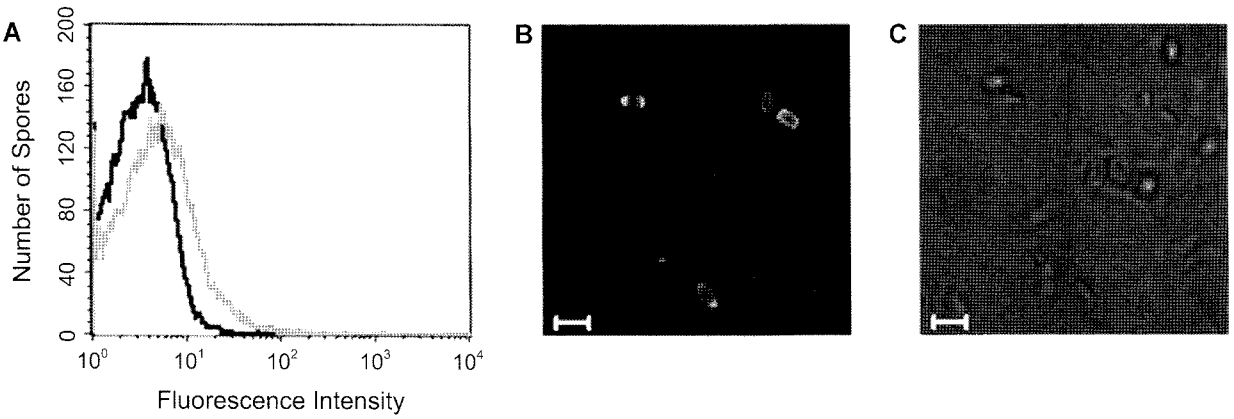


**Fig. 1.** Exosporium display of EGFP.

**A**. Flow cytometry of spores presenting exosporium-displayed β-Gal. Black line, negative control *B. thuringiensis* 4Q7 spores (M=3); Gray line, EGFP-displaying *B. thuringiensis* 4Q7 spores (M=6). **B**. Confocal microscopic image by optical sectioning of EGFP-displaying spores. **C**. Real image of EGFP-displaying spores.

more uniform (Fig. 1A, grey peak) when compared with that of the *B. thuringiensis* 4Q7 as the negative control (Fig. 1A, black peak), indicating that the InhA–EGFP fusion was expressed as an active fusion protein and displayed on the spore surface. The difference in the fluorescence intensity between the control and the EGFP-displaying spores also corresponded to the amount of expressed InhA–EGFP fusion incorporated into the spore surface. To observe the surface-displayed EGFP directly, the EGFP-expressing *B. thuringiensis* 4Q7 spores were analyzed under a confocal microscope (Fig. 1B). Rod-shaped fluorescence from the EGFP was observed on the spores, thereby reconfirming the surface localization of the InhA–EGFP fusion protein on the surface of the *B. thuringiensis* spores. Specific fluorescence signaling of the spores was observed in the EGFP-displaying samples, indicating that the spore-displayed proteins were highly accessible to external reagents, like specific ligands and/or antibodies, allowing binding to the externally expressed EGFP on the spores and making them more likely to be externally exposed.

To determine whether the tetrameric β-Gal enzyme could be displayed in its active form on the surface of the spores, the recombinant *B. thuringiensis* cells were cultivated in DSM medium at 30°C. A sporulation efficiency of ~95% was typically obtained for the recombinant *B. thuringiensis* strains. The spores (~$1.0\times10^8$ CFU/ml) were collected from the cultures in the stationary phase by centrifugation using a urografin gradient (see Materials and Methods). The enzyme activity of the β-Gal displayed on the spores was about $4\times10^2$ U/g (dry weight) of spores based on the hydrolysis of ONPG. Notably, once the InhA-fused β-Gal was expressed and assembled on the spore surface, it was not detached or released into the buffer solution even after several days of incubation. Several physicochemical treatments, such as refrigerated storage, vigorous mixing, and ultrasonic vibration, did not detach the displayed β-Gal.

To confirm and analyze the spore-displayed β-Gal, a flow cytometry analysis was conducted (Fig. 2A). The nonspecific binding of an antibody using the hydrophobic properties of the *B. thuringiensis* spores led to an increase in the fluorescence intensity (red peak) when compared with that of *B. thuringiensis* 4Q7 as the negative control (black peak). Nonetheless, the fluorescence intensity of the purified spores displaying β-Gal showed the highest mean value (green peak). Furthermore, the β-Gal fusion proteins on the *B. thuringiensis* spores were vulnerable to proteinase K (blue peak), indicating that the β-Gal fusion protein was expressed on the spore surface as an active fusion protein.

To define the presence of β-Gal on the spore surface, the vulnerability of the β-Gal on the *B. thuringiensis* spores to several proteases was examined (Fig. 2B). The results revealed that the InhA-displayed β-Gal was vulnerable to several proteases, providing evidence that the β-Gal enzymes were exposed to the outer environment. It is also notable

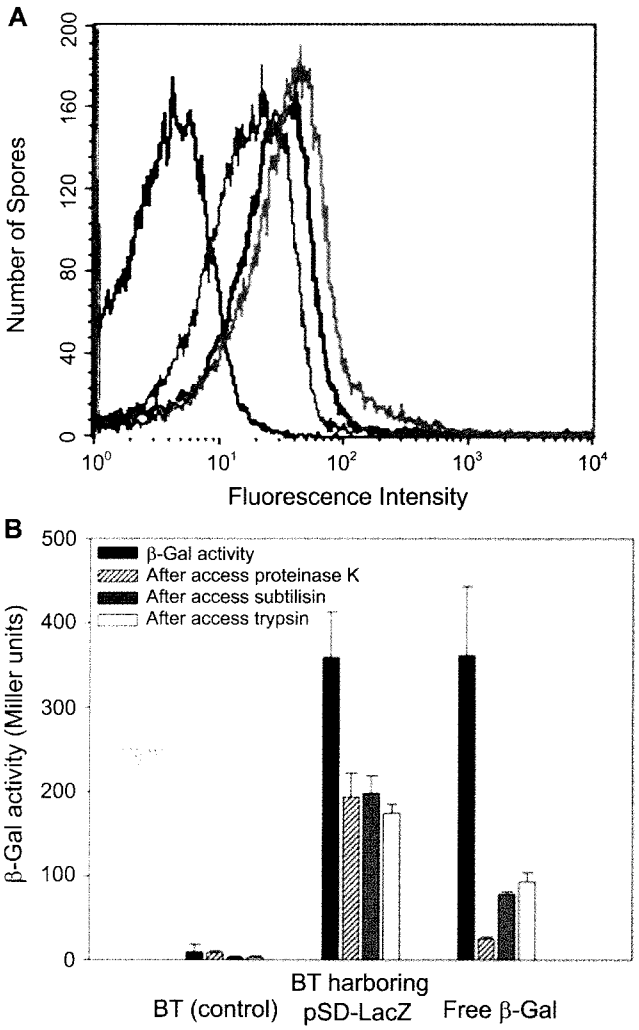


**Fig. 2.** Spore display of β-Gal.
**A**. Flow cytometry of spores presenting exosporium-displayed β-Gal. Black line, negative control *B. thuringiensis* 4Q7 spores (M=4); red line, *B. thuringiensis* 4Q7 spores after treatment with monoclonal β-Gal antibody and secondary FITC-conjugated antibody, respectively (M=22); green line, β-Gal-displaying *B. thuringiensis* 4Q7 spores after treatment with monoclonal β-Gal antibody and secondary FITC-conjugated antibody, respectively (M=41); blue line, β-Gal-displaying *B. thuringiensis* 4Q7 spores after treatment with 200 μg/ml proteinase K for 30 min at 37 °C, and then monoclonal β-Gal antibody and secondary FITC-conjugated antibody, respectively (M=35). **B**. Specific activity of exosporium-displayed β-Gal before (black bars) and after treatment with 200 μg/ml proteinase K (line-patterned bars), subtilisin (grey bars), and trypsin (white bars), respectively. *B. thuringiensis* 4Q7 spores were used as the negative control and free β-Gal as the positive control. All the samples were incubated at 37°C for 30 min in a water bath.

that the InhA-displayed β-Gal became more resistant to proteases than the free β-Gal, indicating that the enzymatic activity of the surface-displayed protein was maintained even under the presence of protease. Researchers have previously attempted to use the spores themselves as biocatalysts [20] and spore-displayed enzymes as a carrier of recombinant enzymes [16]. Thus, improving the resistance

towards proteases may facilitate the design of bioconversion processes [1].

**Immunogold Analysis of Spore-Displayed Protein**

Transmission electron microscopy (TEM) of the β-Gal-displaying *B. thuringiensis* spores exposed to gold colloid particles (10 nm) was used to observe the display more precisely (Fig. 3). The TEM analysis of the gold colloid-bound β-Gal-displaying spores yielded images of a balloon-like exosporium completely covering the *B. thuringiensis* spore (Fig. 3), indicating that the immunogold-bound exosporium had partially split off from the original spore, perhaps due to overgrowth of the artificial exosporium derived from the overexpression of the InhA–β-Gal fusion protein and a weakened structure of the exosporium caused by the fusion of the recombinant protein. Additionally, this phenomenon indicated that the fusion protein was vulnerable to attack by an InhA metalloprotease or mechanical damage during the sample preparation step for TEM imaging. Nonetheless, the images confirmed that the β-Gal fusion proteins were displayed on the surface of the spores.

It should also be noted that some trouble was experienced reproducing the EM results. This may have been due to the weak structure of the balloon-like exosporium, making the sample preparation difficult while maintaining the original structure of the *B. thuringiensis* spores. The fusion between the InhA and the target protein may also have been the underlying cause for the unstable structure of the exosporium. Another possibility is that fusing with InhA may affect the stability of the recombinant fusion protein, as InhA is a zinc metalloprotease, the major function of which is to selectively degrade antimicrobial peptides [5, 7]. Therefore, to further develop the present system, it is suggested that InhA could be improved by directed-mutagenesis of its protease-like domain.

This study presented a novel exosporium protein-based surface-display system on *B. thuringiensis* spores. Monomeric GFP and tetrameric β-Gal were both correctly displayed. As a delivery vehicle, the exosporium surrounding the

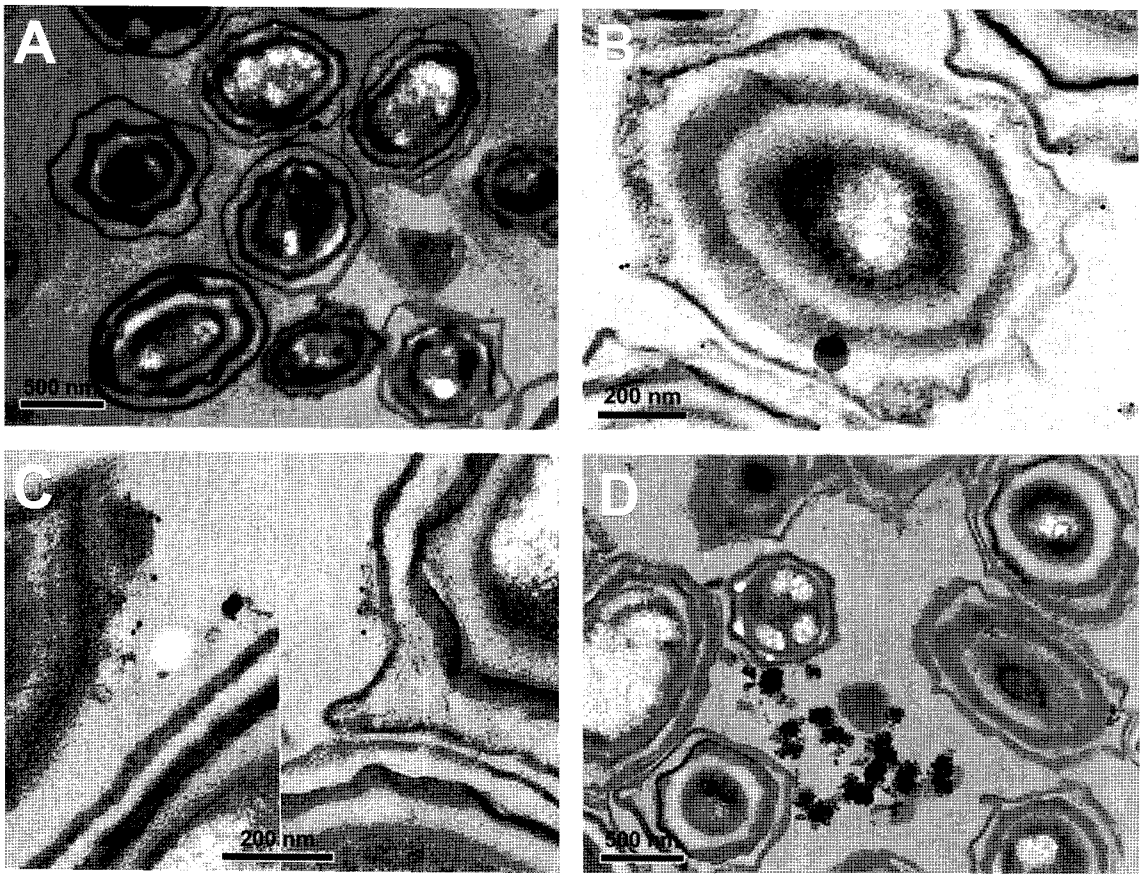


**Fig. 3.** Transmission electron microscopy (TEM) analysis of InhA-displayed spores using immunogold.
**A**. TEM image of control spores treated with β-Gal antibody and secondary gold-antibody. Scale bar represents 500 nm. **B–C**. Selected TEM images (80 kV, ×100,000) of InhA-displayed β-Gal. Scale bars represent 200 nm. **D**. TEM image (80 kV, ×30,000) of InhA-displayed β-Gal and split InhA-fused β-Gal bound with immunogold. Scale bar represents 500 nm. Gold nanoparticles (nominal diameter of 10 nm) were observed after sequential treatment with the monoclonal β-Gal antibody and secondary gold-conjugated antibody.

entire surface of spores provides a suitable surface for the display of heterologous proteins; thus, it is expected that the results of this study can be extended to other exosporium proteins, including other exosporal components in *B. thuringiensis*, the Exs series in *B. cereus,* and BclA in *B. anthracis* [5, 26, 28]. Consequently, exosporium protein-based spore-display systems may prove useful in the development of an improved version of molecular display: covering the whole surface. *Bacillus* spores are resistant to solvents, extreme pHs and temperatures, and high concentrations of salt [22]. Therefore, by taking advantage of the natural robustness of these spores, the proposed spore-display system can be broadly applied to the fields of oral vaccines, whole-cell biocatalysis, high-throughput screening of protein libraries, and other applications [24].

## Acknowledgments

This work was supported by the Korean Systems Biology Research Program (M10309020000-03B5002-00000) of the Korean Ministry of Education, Science, and Technology (T.J.P. and S.Y.L.), the IT Leading R&D Support Project from the Korean Ministry of Knowledge Economy through IITA (T.J.P. and S.Y.L.), and the National Research Laboratory Program of the Korean Ministry of Commerce, Industry, and Energy (S.K.C, H.C.J., and J.G.P.). The authors would also like to thank Hye Jin Choi for her assistance with the TEM imaging (Korea Basic Science Institute, Korea).

### References

1. Arnold, F. H. and A. A. Volkov. 1999. Directed evolution of biocatalysts. *Curr. Opin. Chem. Biol.* **3:** 54–59.
2. Boder, E. T. and K. D. Wittrup. 1997. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15:** 553–557.
3. Charlton, S., A. J. Moir, L. Baillie, and A. Moir. 1999. Characterization of the exosporium of *Bacillus cereus*. *J. Appl. Microbiol.* **87:** 241–245.
4. Daugherty, P. S., G. Chen, M. J. Olsen, B. L. Iverson, and G. Georgiou. 1998. Antibody affinity maturation using bacterial surface display. *Protein Eng.* **11:** 825–832.
5. Fedhila, S., N. Patricia, and D. Lereclus. 2002. The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected *via* the oral route. *J. Bacteriol.* **184:** 3296–3304.
6. Georgiou, G., C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson, and R. Curtiss 3rd. 1997. Display of heterologous proteins on the surface of microorganisms: From the screening of combinatorial libraries to live recombinant vaccines. *Nat. Biotechnol.* **15:** 29–34.
7. Grandvalet, C., M. Gominet, and D. Lereclus. 2001. Identification of genes involved in the activation of the *Bacillus thuringiensis* *inhA* metalloprotease gene at the onset of sporulation. *Microbiology* **147:** 1805–1813.
8. Henriques, A. O. and C. P. Moran Jr. 2007. Structure, assembly, and function of the spore surface layers. *Annu. Rev. Microbiol.* **61:** 555–588.
9. Hoa, N. T., L. Baccigalupi, A. Huxham, A. Smertenko, P. H. Van, S. Ammendola, E. Ricca, and A. S. Cutting. 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Appl. Environ. Microbiol.* **66:** 5241–5247.
10. Isticato, R., G. Cangiano, H. T. Tran, A. Ciabattini, D. Medaglini, M. R. Oggioni, M. De Felice, G. Pozzi, and E. Ricca. 2001. Surface display of recombinant proteins on *Bacillus subtilis* spores. *J. Bacteriol.* **183:** 6294–6301.
11. Jung, H. C., J. M. Lebeault, and J. G. Pan. 1998. Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. *Nat. Biotechnol.* **16:** 576–580.
12. Kim, H., M. Hahn, P. Grabowski, D. C. McPherson, M. M. Otte, R. Wang, C. C. Ferguson, P. Eichenberger, and A. Driks. 2006. The *Bacillus subtilis* spore coat protein interaction network. *Mol. Microbiol.* **59:** 487–502.
13. Kim, J.-H., C. Roh, C.-W. Lee, D. Kyung, S.-K. Choi, H.-C. Jung, J.-G. Pan, and B.-G. Kim. 2007. Bacterial surface display of $GFP_{UV}$ on *Bacillus subtilis* spores. *J. Microbiol. Biotechnol.* **17:** 677–680.
14. Kim, J. H., C. S. Lee, and B. G. Kim. 2005. Spore-displayed streptavidin: A live diagnostic tool in biotechnology. *Biochem. Biophys. Res. Commun.* **331:** 210–214.
15. Kim, Y. S., H. C. Jung, and J. G. Pan. 2000. Bacterial cell surface display of an enzyme library for selective screening of improved cellulase variants. *Appl. Environ. Microbiol.* **66:** 788–793.
16. Kwon, S. J., H.-C. Jung, and J.-G. Pan. 2007. Transgalactosylation in a water-solvent biphasic reaction system with β-galactosidase displayed on the surface of *Bacillus subtilis* spores. *Appl. Environ. Microbiol.* **73:** 2251–2256.
17. Lee, J. S., K. S. Shin, J. G. Pan, and C. J. Kim. 2000. Surface-displayed viral antigens on *Salmonella* carrier vaccine. *Nat. Biotechnol.* **18:** 645–648.
18. Lee, S. Y., J. H. Choi, and Z. Xu. 2003. Microbial cell-surface display. *Trends Biotechnol.* **21:** 45–52.
19. Mauriello, E. M., H. Duc le, R. Isticato, G. Cangiano, H. A. Hong, M. De Felice, E. Ricca, and S. M. Cutting. 2004. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. *Vaccine* **22:** 1177–1187.
20. Murata, K. 1993. Use of microbial spores as a biocatalyst. *Crit. Rev. Biotechnol.* **13:** 173–193.
21. Nicholson, W. L. and P. Setlow. 1990. Sporulation, germination and outgrowth, pp. 391–429. *In* C. R. Harwood and S. M. Cutting (eds.), *Molecular Biological Methods for Bacillus*. John Wiley & Sons Ltd., West Sussex, U.K.
22. Nicholson, W. J., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev.* **64:** 548–572.
23. Park, T. J., K. B. Lee, S. J. Lee, J. P. Park, Z. W. Lee, S. K. Choi, *et al.* 2004. Micropatterns of spores displaying heterologous proteins. *J. Am. Chem. Soc.* **126:** 10512–10513.

24. Ricca, E. and S. M. Cutting. 2003. Emerging applications of bacterial spores in nanobiotechnology. *J. Nanobiotechnol.* **1**: 6.
25. Sambrook, J. and D. Russell. 2001. *Molecular Cloning: A Laboratory Manual,* 3rd Ed. Cold Spring Harbor Laboratory Press, New York, NY.
26. Steichen, C., P. Chen, J. F. Kearney, and C. L. Turnbough Jr. 2003. Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *J. Bacteriol.* **185:** 1903–1910.
27. Sylvestre, P., E. Couture-Tosi, and M. Mock. 2002. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Mol. Microbiol.* **45:** 169–178.
28. Todd, S. J., A. J. Moir, M. J. Johnson, and A. Moir. 2003. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *J. Bacteriol.* **185:** 3373–3378.
29. Xu, Z. and S. Y. Lee. 1999. Display of polyhistidine peptides on the *Escherichia coli* cell surface by using outer membrane protein C as an anchoring motif. *Appl. Environ. Microbiol.* **65:** 5142–5147.