

## Surface Display of Recombinant Proteins on *Bacillus thuringiensis* Spores

Cheng Du,<sup>1\*</sup> Wing C. Chan,<sup>1</sup> Timothy W. McKeithan,<sup>2</sup>  
and Kenneth W. Nickerson<sup>3</sup>

Department of Pathology and Microbiology<sup>1</sup> and Department of Internal Medicine,<sup>2</sup> University of  
Nebraska Medical Center, Omaha, Nebraska 68198, and School of Biological Sciences,  
University of Nebraska—Lincoln, Lincoln, Nebraska 68588<sup>3</sup>

Received 31 August 2004/Accepted 15 December 2004

**The insecticidal protoxin from *Bacillus thuringiensis* has been shown to be a major component of the spore coat. We have developed a novel surface display system using *B. thuringiensis* spores in which the N-terminal portion of the protoxin is replaced with a heterologous protein. The expression vector with a sporulation-specific promoter was successfully used to display green fluorescent protein and a single-chain antibody (scFv) gene that encodes anti-4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (anti-phOx) antibody. The spores that carry the anti-phOx antibody can bind to phOx specifically.**

Biological surface display systems, such as bioabsorbents and biocatalysts, have been applied in many areas, including the screening of novel binding partners, delivery of vaccines and drugs, and production of active enzymes and antibodies for cleanup of industrial and environmental pollution (13). Systems have been developed to display heterologous proteins on the surfaces of bacteriophage (22), *Escherichia coli* (2, 5, 12), and *Saccharomyces cerevisiae* (4). Bacterial spores, a dormant form of living cells known for their resistance to harsh environments, represent a unique opportunity for surface display. Bacterial-spore display systems may provide a durable supporting matrix similar to that of chemical polymer beads, which can easily and economically be produced in large quantity. A surface display system using CotB, a component of the *Bacillus subtilis* spore coat, has been developed and used for producing heterologous antigens (14).

During sporulation, *Bacillus thuringiensis* cells form protein crystals of insecticidal protein toxins (the delta-endotoxins), which have been used as biological pesticides. Many studies have shown that the 130-kDa *B. thuringiensis* protoxin from the Cry1Ac subgroup is also a major component of the spore coat. Protein extracts from *B. thuringiensis* spores react with antiprototoxin antibody and have insecticidal activity like that of the crystal protein (9). An electron microscopy study showed that the protoxin exists in the spore coat layer (23) but only in *cry*<sup>+</sup> strains (which produce the toxins), not in *cry* mutant strains, which have lost the toxin-encoding plasmids and do not produce the crystals (3). We previously proposed a model in which the N-terminal “business end” of the protoxin is exposed on the spore surface and the C-terminal region anchors the protoxin inside the spore coat (10).

**Plasmids.** The *B. thuringiensis* toxin sporulation-specific promoter region from strain HD73 (GenBank accession number M11068; hereinafter, nucleotide numbers refer to this se-

quence) has been determined (1). It contains two individual promoter sites, Bt1 and Bt2. A 241-bp NsiI fragment containing both promoters was inserted into the PstI site of the shuttle vector pHT3101 (16). The resulting plasmid, pCD2, also encodes the first 11 amino acids of Cry1Ac toxin (Fig. 1A). Various C-terminal regions of the protoxin were amplified by PCR, fused with full-length green fluorescent protein (GFP) from plasmid pGreen Lantern-1 (Invitrogen), and inserted in frame into pCD2. The following individual constructs, shown in Fig. 1C, contain the indicated *B. thuringiensis* protoxin C-terminal regions: (i) Bt3, GFP alone, no fusion partner; (ii) Bt5, nucleotides (nt) 2202 to 3217; (iii) Bt6, nt 2565 to 2980; (iv) Bt7, nt 3217 to 3925 (stop codon); (v) Bt8, nt 2278 to 3925; (vi) Bt9, nt 2278 to 2980; (vii) Bt10, nt 2278 to 3685; and (viii) Bt12, nt 2566 to 3685. Detailed maps for these constructs are available on request. Plasmid pCD4 was made by inserting the fragment of nt 2565 to 2980 (415 bp) into the KpnI and EcoRI sites of pCD2 (Fig. 1B).

**Electroporation.** *B. thuringiensis* electroporation was performed as described by Macaluso and Mettus (17). *B. thuringiensis cry* mutant strains were grown in brain heart infusion medium plus 0.5% glycerol overnight at 30°C. The culture was diluted 1:20 into prewarmed fresh medium and grown for 1 h. The culture was pelleted and washed twice with ice-cold electroporation buffer (0.625 M sucrose, 1 mM MgCl<sub>2</sub>). The cells were resuspended in electroporation buffer at half the volume of the original culture. Eight-tenths milliliter of cells and 2 μl of plasmid (about 0.1 to 0.2 μg DNA, purified with a QIAGEN Qiaprep mini column) were mixed in a 4-mm cuvette and incubated on ice for 5 min. Electroporation was carried out in a Bio-Rad GenePulse II with settings of 1.3 kV, 25 μF, and 50 Ω. The cells were then mixed with 1.6 ml of brain heart infusion medium plus 0.5% glycerol and incubated at 30°C for 1 h, and 5 to 100 μl was plated on LB plates with 25 μg/ml erythromycin.

**Bacterial strains.** *E. coli* strain DH5α was used for routine plasmid manipulation, and strain JM110 (*dam dcm* mutant) was used to produce plasmids for *B. thuringiensis* electropora-

\* Corresponding author. Mailing address: Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198. Phone: (402) 559-7921. Fax: (402) 559-4077. E-mail: cdu@unmc.edu.

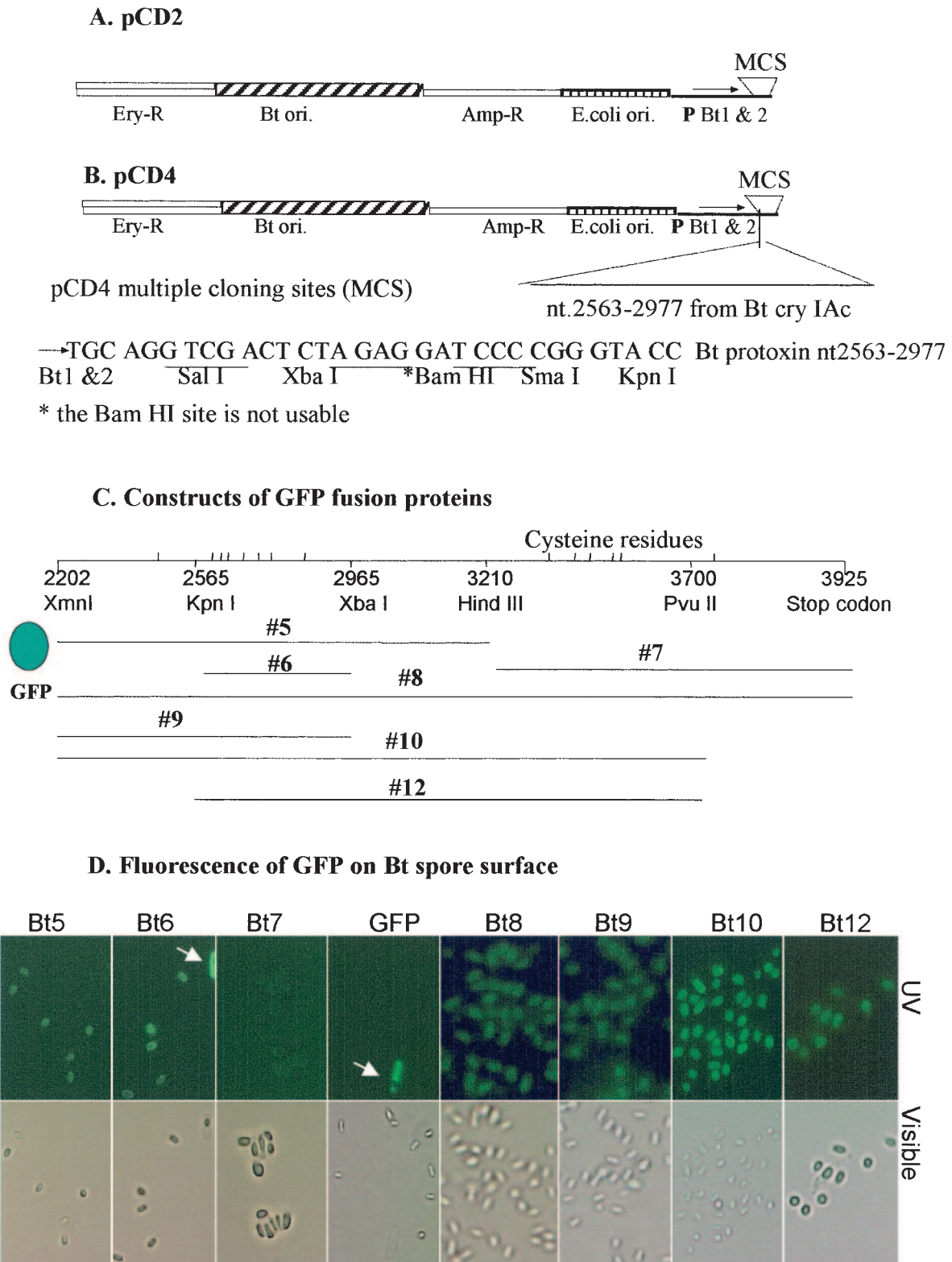


FIG. 1. Display of GFP on the *B. thuringiensis* spore surface. (A) Vector pCD2 (6.6 kb) was derived from pHT3101 by inserting the *B. thuringiensis* toxin promoter region into the PstI site. This plasmid can grow in both *E. coli* (with ampicillin selection [Amp-R]) and *B. thuringiensis* (with erythromycin selection [Ery-R]). The inserted promoters (P) are sporulation specific. (B) pCD4, derived from pCD2, is a *B. thuringiensis* spore surface display vector. A 415-bp fragment from the *cryIAC* gene was inserted downstream of multiple cloning sites. Fusion proteins can be displayed on the *B. thuringiensis* spore surface anchored by the portion derived from the *B. thuringiensis* protoxin. (C) GFP fusion proteins are shown schematically. The approximate nucleotide numbers and restriction sites are shown. The positions of cysteine residues are also shown. (D) Expression of various GFP fusion proteins on the surfaces of *B. thuringiensis* spores. The white arrows indicate mother cells.

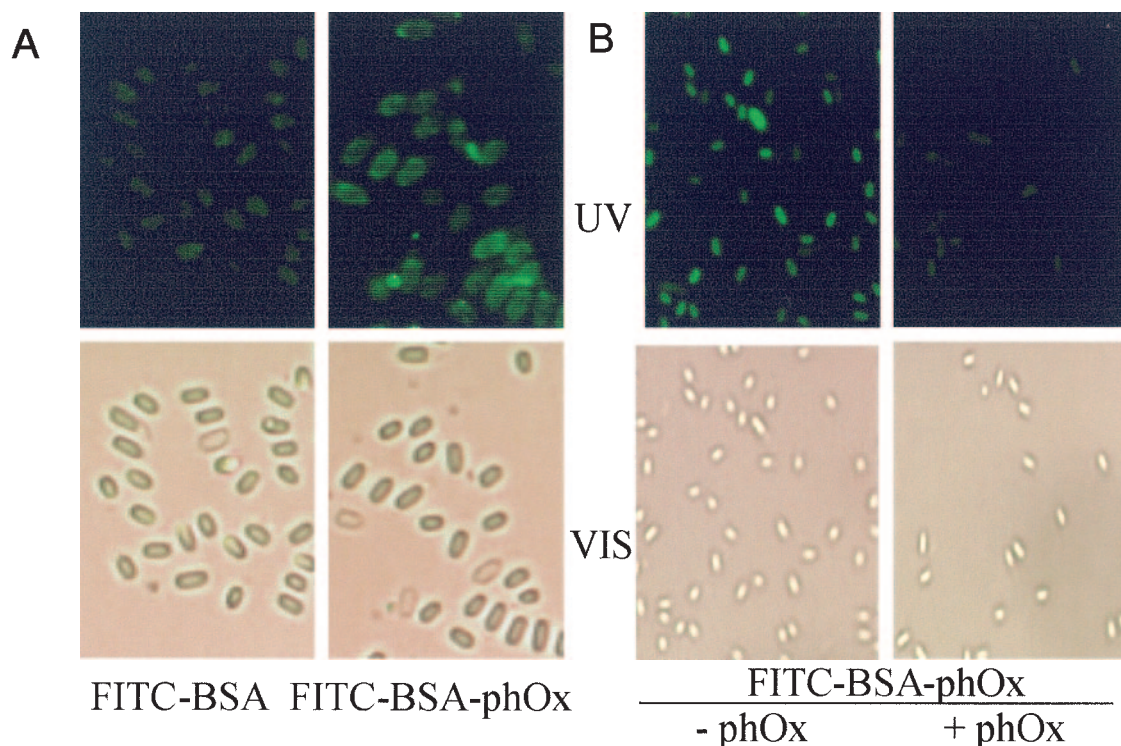


FIG. 2. Anti-phOx antibody displayed on the *B. thuringiensis* spore surface can bind antigen. After being washed and blocked with BSA, spores that carry anti-phOx antibody were incubated with either BSA-FITC conjugate or BSA-FITC-phOx (A). Fluorescence (top) and visible light (VIS) (bottom) images are shown for the resulting spores. In the presence of excess phOx, the binding of FITC-BSA-phOx to spores was abolished (B).

tion (17). *B. thuringiensis* crystal-free ( $Cry^-$ ) strains 4D7, 4D8, 4D9, 4D11, 4D22, 4E5, 4Q7, and 4Q8 (all kindly provided by D. R. Zeigler of the Bacillus Genetics Stock Center) have been tested in spore display.

***B. thuringiensis* spore surface display.** After plasmid transformation of the  $Cry^-$  strain 4D7, the cells were grown in GYS medium to promote sporulation (20). The spores were washed with a high-salt buffer, TBSE1 (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 5 mM EDTA) to remove attached soluble proteins (19).

The fluorescence of each GFP construct is shown in Fig. 1D. When GFP is expressed alone (construct Bt3) without any C-terminal part of *B. thuringiensis* protoxin, the protein level of GFP is high in the sporulating mother cell but not on the spore surface (Fig. 1D). This result also indicates that the promoters used in pCD2 have strong transcription activity and that the GFP protein is stable and functional as judged by the intensity of its green fluorescence.

Since the cleavage site of Cry1Ac protoxin by proteases is not well defined, two start positions of the C termini were chosen. As seen in Fig. 1C, the first start is at nt 2202, within conserved block 5 of the active toxin (21). The second start is at nt 2278, which is about 20 amino acid residues downstream of block 5. Construct Bt8 included the full-length C terminus of the protoxin and gave moderate fluorescence intensity on the spore surface (Fig. 1D). Other constructs, Bt5, -6, -9, -10, and -12 also provided surface fluorescence. Construct Bt10 yielded the brightest color and thus the highest expression level of GFP (Fig. 1D). In contrast, construct Bt7 did not yield fluorescence on the spore surface and thus lacked the region nec-

essary for anchoring. We conclude that construct Bt6, which includes nt 2565 to 2980 (a 415-bp fragment), contains the minimal region required for displaying foreign proteins. Thus, expression vector pCD4 was constructed by inserting the 415-bp fragment of *B. thuringiensis* protoxin under the control of the Bt1 and Bt2 promoters in pCD2. It is noted that this 415-bp region encodes a cysteine-rich polypeptide (7 cysteine residues in 138 amino acids).

Constructs Bt6 and Bt10 were also transformed by electroporation into several other  $Cry^-$  strains: 4D8, 4D9, 4D11, 4D22, 4E5, 4Q7, and 4Q8. All strains, except 4Q7 and 4Q8, which were not transformable with our electroporation protocol, successfully displayed GFP on their surfaces (data not shown).

**Presenting a functional single-chain antibody (scFv) on the *B. thuringiensis* spore surface.** DNA encoding a known scFv antibody against the hapten 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx) (6, 18) was amplified from plasmid pHOOK\_3 (Invitrogen, Carlsbad, CA) by PCR and cloned in frame into the SalI and KpnI sites of pCD4. The primers used were 5'-GCGTTCGACCTATCCATATGATGTTCC-3' (forward) and 5'-GGGGTACCCGTTTCAGCTCCAG-3' (reverse). The resulting plasmid, pCD4-phOx, was transformed into strain 4D7.

Bovine serum albumin (BSA), in 0.1 M  $NaHCO_3$ , pH 8.5, at 1 mg/ml, was mixed with fluorescein isothiocyanate (FITC [Sigma Chemicals] dissolved in dimethyl sulfoxide [DMSO]). After incubation at room temperature for 1 h, the mixture was divided into two equal parts. phOx (from Sigma Chemicals,

dissolved in DMSO) was added to one portion and incubated for another hour at room temperature. The other portion was treated with DMSO only. One molar glycine was added in excess to stop the reaction. Both samples were then dialyzed against TBSE2 (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA) to remove unconjugated FITC and phOx. The FITC-BSA and FITC-BSA-phOx conjugates were aliquoted and stored at  $-20^{\circ}\text{C}$ . Using a molar  $\epsilon_{495\text{ nm}}$  of  $79,000\text{ M}^{-1}\text{ cm}^{-1}$  for FITC and a  $\epsilon_{322\text{ nm}}$  of  $27.54\text{ mM}^{-1}\text{ cm}^{-1}$  for phOx (according to the manufacturer), the labeling ratios of FITC and phOx were calculated as 1.1 and 15.1 per BSA molecule, respectively.

*B. thuringiensis* spores with anti-phOx on their surfaces were incubated with BSA (1 mg/ml in TBSE1) to block nonspecific binding. FITC-BSA or FITC-BSA-phOx was added to the spores and incubated on ice for 2 h. In a separate experiment, an approximately 200-fold molar excess of free phOx (quenched by preincubation with a Tris-glycine solution) was added along with FITC-BSA-phOx to the anti-phOx spores. The spores were then washed with TBSE2 five times before microscopic examination.

As seen in Fig. 2, no fluorescence was observed with FITC-BSA, indicating that it was not recognized by anti-phOx antibody on the *B. thuringiensis* spores. Spores incubated with FITC-BSA-phOx had visible surface fluorescence, indicating that the spores bound the phOx hapten via surface anti-phOx antibody. In the presence of excess free phOx, the fluorescence was competed off from the anti-phOx spores. We conclude that the scFv anti-phOx antibody can be expressed and displayed on the *B. thuringiensis* spore surface in a functional conformation and that the antigen (FITC-BSA-phOx) can gain access to the antibody on the spore surface. Considering that the size of BSA is 66 kDa, spatial barriers on the spore surface that might potentially block antigen access are unlikely to be a big concern.

The *B. thuringiensis* spore display system has several advantages over a similar *B. subtilis* system. The *B. subtilis* surface display system based on CotB protein requires endogenous CotB protein expression. No fusion protein can be displayed in a CotB<sup>-</sup> strain (14). This dependence may cause competition for expression and display of fusion proteins with the wild-type CotB protein. In fact, the expression of the wild-type CotB protein on the spores is much higher than that of fusion proteins (14). Since the natural *B. thuringiensis* toxin genes are located on dispensable plasmids, fusion proteins need not compete with the native toxin for expression and display using the *B. thuringiensis* display system. In addition, more complete sporulation can be achieved with *B. thuringiensis* than with *B. subtilis* (98 to 100% versus about 70%).

Unequal proliferation tends to introduce biases in peptide libraries during cell propagation (7, 8, 11, 15). The use of inducible promoters will help to minimize this distortion because the protein library need not be expressed during propagation. The Bt1 and Bt2 promoters used in the expression vector are activated only during sporulation. Manipulation of the growth medium controls the sporulation process, the activities of the promoters, and thus also the expression of the recombinant proteins.

In summary, we have developed a surface display system on *B. thuringiensis* spores. In this system, the expression of heterologous proteins is controlled by sporulation-specific promot-

ers. The minimal region of the anchoring partner was also defined. This system may prove valuable for screening for ligand binding under highly stringent conditions.

We thank D. R. Zeigler of the Bacillus Genetics Stock Center for all *B. thuringiensis* Cry<sup>-</sup> strains and B. A. Federici for plasmid pHT3101 and a full-length Cry1Ac clone.

This work was partially supported by a College of Medicine Research Grant Award, from the University of Nebraska Medical Center, to C.D.

## REFERENCES

- Adams, L. F., K. L. Brown, and H. R. Whiteley. 1991. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. *J. Bacteriol.* **173**:3846-3854.
- Agterberg, M., H. Adriaanse, A. van Bruggen, M. Karperien, and J. Tommassen. 1990. Outer-membrane PhoE protein of *Escherichia coli* K-12 as an exposure vector: possibilities and limitations. *Gene* **88**:37-45.
- Aronson, A. I., D. J. Tyrell, P. C. Fitz-James, and L. A. Bulla, Jr. 1982. Relationship of the syntheses of spore coat protein and parasporal crystal protein in *Bacillus thuringiensis*. *J. Bacteriol.* **151**:399-410.
- Boder, E. T., and K. D. Wittrup. 1997. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**:553-557.
- Charbit, A., A. Molla, W. Saurin, and M. Hofnung. 1988. Versatility of a vector for expressing foreign polypeptides at the surface of gram-negative bacteria. *Gene* **70**:181-189.
- Clackson, T., H. R. Hoogenboom, A. D. Griffiths, and G. Winter. 1991. Making antibody fragments using phage display libraries. *Nature* **352**:624-628.
- Daugherty, P. S., M. J. Olsen, B. L. Iverson, and G. Georgiou. 1999. Development of an optimized expression system for the screening of antibody libraries displayed on the *Escherichia coli* surface. *Protein Eng.* **12**:613-621.
- de Bruin, R., K. Spelt, J. Mol, R. Koes, and F. Quattrocchio. 1999. Selection of high-affinity phage antibodies from phage display libraries. *Nat. Biotechnol.* **17**:397-399.
- Delafield, F. P., H. J. Somerville, and S. C. Rittenberg. 1968. Immunological homology between crystal and spore protein of *Bacillus thuringiensis*. *J. Bacteriol.* **96**:713-720.
- Du, C., and K. W. Nickerson. 1996. *Bacillus thuringiensis* HD-73 spores have surface-localized Cry1Ac toxin: physiological and pathogenic consequences. *Appl. Environ. Microbiol.* **62**:3722-3726.
- Feldhaus, M. J., R. W. Siegel, L. K. Opresko, J. R. Coleman, J. M. Feldhaus, Y. A. Yeung, J. R. Cochran, P. Heinzelman, D. Colby, J. Swers, C. Graff, H. S. Wiley, and K. D. Wittrup. 2003. Flow-cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library. *Nat. Biotechnol.* **21**:163-170.
- Francisco, J. A., C. F. Earhart, and G. Georgiou. 1992. Transport and anchoring of beta-lactamase to the external surface of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **89**:2713-2717.
- Georgiou, G., C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson, and R. Curtiss III. 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.
- Isticato, R., G. Cangiano, H. T. Tran, A. Ciabattini, D. Medagliani, 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.
- Krebber, A., J. Burmester, and A. Pluckthun. 1996. Inclusion of an upstream transcriptional terminator in phage display vectors abolishes background expression of toxic fusions with coat protein g3p. *Gene* **178**:71-74.
- Lereclus, D., O. Arantes, J. Chauvaux, and M. Lecadet. 1989. Transformation and expression of a cloned delta-endotoxin gene in *Bacillus thuringiensis*. *FEMS Microbiol. Lett.* **51**:211-217.
- Macaluso, A., and A. M. Mettus. 1991. Efficient transformation of *Bacillus thuringiensis* requires nonmethylated plasmid DNA. *J. Bacteriol.* **173**:1353-1356.
- Marks, J. D., H. R. Hoogenboom, T. P. Bonnett, J. McCafferty, A. D. Griffiths, and G. Winter. 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **222**:581-597.
- Nickerson, K. W., and J. D. Swanson. 1981. Removal of contaminating proteases from *Bacillus thuringiensis* parasporal crystals by density gradient centrifugation in NaBr. *Eur. J. Appl. Microbiol. Biotechnol.* **13**:213-215.
- Nickerson, K. W., and L. A. Bulla, Jr. 1974. Physiology of sporeforming

- bacteria associated with insects: minimal nutritional requirements for growth, sporulation, and parasporal crystal formation of *Bacillus thuringiensis*. *Appl. Microbiol.* **28**:124–128.
21. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**:775–806.
  22. **Scott, J. K., and G. P. Smith.** 1990. Searching for peptide ligands with an epitope library. *Science* **249**:386–390.
  23. **Short, J. A., P. D. Walker, R. O. Thomson, and H. J. Somerville.** 1974. The fine structure of *Bacillus finitimus* and *Bacillus thuringiensis* spores with special reference to the location of crystal antigen. *J. Gen. Microbiol.* **84**: 261–276.