Surface Display of Recombinant Proteins on Bacillus subtilis Spores

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We developed a novel surface display system based on the use of bacterial spores. A protein of the Bacillus subtilis spore coat, CotB, was found to be located on the spore surface and used as fusion partner to express the 459-amino-acid C-terminal fragment of the tetanus toxin (TTFC). Western, dot blot and fluorescent-activated cell sorting analyses were used to monitor TTFC surface expression on purified spores. We estimated that more than 1.5 × 10^3 TTFC molecules were exposed on the surface of each spore and recognized by TTFC-specific antibodies. The efficient surface presentation of the heterologous protein, together with the simple purification procedure and the high stability and safety record of B. subtilis spores, makes this spore-based display system a potentially powerful approach for surface expression of bioactive molecules.

Presentation of heterologous proteins in a biologically active form is an important task with potential applications (11, 32) in a variety of fields ranging from live-vaccine development (19–21, 24) to treatment of microbial infections (3), peptide library screening (4), and biocatalyst or biosorbent development (25, 31).

Several approaches have been undertaken to develop efficient display systems expressing heterologous polypeptides on the surface of cells (4, 11, 29, 32) and viruses (2, 7, 26, 30). In gram-negative bacteria, various surface proteins such as outer membrane proteins LamB and OmpA and lipoproteins have been exploited as fusion partners to express bacterial and viral antigens and induce specific antibody response in animals immunized with the recombinant bacteria (19, 29).

Several gram-positive bacteria have also been used for the expression of heterologous proteins, mainly antigenic determinants for the induction of both local and systemic antibody response in animal models (3, 20, 21, 24). In addition, many gram-positive species have been considered for purposes other than vaccine development. Because of the thicker cell wall, gram-positive bacteria are viewed as preferential candidates over gram-negative bacteria for the development of bacterial biocatalysts and whole-cell adsorbents (19, 29).

Here we report a novel surface display system based on the use of bacterial spores. There are many potential advantages with the utilization of spores: (i) a high stability of the expression system is ensured by the well-documented resistance and stability of the bacterial spore (9, 14); (ii) a good safety record is ensured by the use of spores of species including Bacillus subtilis, B. clausii, B. coagulans, B. cereus, and B. natto as food additives in human and animal food preparations and as prescription or nonprescription products for the treatment of gastrointestinal disorders (12, 15); (iii) simple and economic production of large amounts of spores is ensured by commercially available and commonly used procedures for industrial-scale production and commercialization of several spore-based products (12, 15).

Among the various Bacillus species, B. subtilis offers additional advantages due to the detailed knowledge of its spore structure (9, 14) and the availability and ease of advanced genetic tools (5) and genomic data (17) that facilitate the construction of recombinant spores. B. subtilis spores are surrounded by a coat, a proteinaceous structure organized into two layers and composed of at least 20 polypeptides (9, 14). Some of these, like CotA, CotB, CotC (8), CotF (6), and CotG (27), have been associated with the outer part of the coat and are referred to as outer coat proteins (9, 14), but for all of them the exact location within the coat and the protein domain required for external localization are not known.

Here we show that the previously identified outer coat component CotB (8) (Fig. 1A) is localized on the spore surface, and based on this, we report the use of CotB as a fusion partner for surface display on B. subtilis spores. As a model system, we expressed the 459-amino-acid C-terminal fragment of the tetanus toxin (TTFC) (13). This work provides the first evidence that a heterologous protein can be expressed on the surface of a bacterial spore and points to this peculiar cell form as a novel and potentially powerful system to display bioactive molecules.

**MATERIALS AND METHODS**

**Bacterial strains and transformation.** B. subtilis strains utilized are listed in Table 1. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of B. subtilis competent cells were performed with Escherichia coli strain DH5α (28). Bacterial strains were transformed by previously described procedures: CaCl2-mediated transformation of E. coli competent cells (28) and two-step transformation of B. subtilis (5).

**Construction of gene fusions.** The general strategy for the construction of the gene fusions is shown in Fig. 1B. Fragments of cotB DNA were PCR amplified from the B. subtilis chromosome priming the amplification with the synthetic oligonucleotides shown in Fig. 1B and listed in Table 2. The PCR products were

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visualized on ethidium bromide-stained agarose gels and gel purified by the QIAquick gel extraction kit (Qiagen) as specified by the manufacturer.

(i) Fusion A. A purified 1,071-bp DNA fragment originating from the amplification of B. subtilis chromosomal DNA with B1 and B3 oligonucleotides (Table 2) was sequentially digested with SphI and BglII and cloned in frame to the 5′/H11032 end of the tetC gene carried by plasmid pGEM-TTFC (24), yielding plasmid pNS4.

(ii) Fusion B. A purified 246-bp DNA fragment originating from the amplification of B. subtilis chromosomal DNA with B1 and B6 oligonucleotides (Table 2) was digested with SphI and BamHI and cloned into plasmid pGEM-TTFC (20) in frame with the 5′/H11032 end of the tetC gene. The plasmid thus obtained was cleaved with BglII and XhoI and used to clone to the 3′/H11032 end of tetC a second PCR fragment of 825 bp, originating from amplification of the B. subtilis chromosome with B7 and B3 oligonucleotides (Table 2). The resulting plasmid was named pNS16.

(iii) Fusion C. A purified DNA fragment of 123 bp originating from the amplification of B. subtilis chromosomal DNA with B4 and B5 oligonucleotides (Table 2) was digested with SalI and BglII and ligated into plasmid pNS4 (fusion A), yielding plasmid pNS8.

On digestion with enzymes SphI and SalI, pNS4 and pNS16 released a 2,474-bp DNA fragment while pNS9 released a 2,597-bp fragment, all containing the cotB gene region fused to tetC. These fragments were gel purified as described above and ligated into plasmid pDG364 (5) previously digested with the same two enzymes. E. coli competent cells were transformed with the ligation mixture, and the selected ampicillin-resistant clones were screened by restriction analysis of their plasmids. Individual clones for each transformation were selected, named pNS6, pNS17, and pNS9, respectively, and used to determine the nucleotide sequence of the inserted DNA.

Chromosomal integration. Plasmids pNS6, pNS9, and pNS17 were linearized by digestion with PstI and used to transform competent cells of the B. subtilis strain PY79. Cm’ clones were the result of double-crossover recombination, resulting in the interruption of the nonessential amylE gene on the B. subtilis chromosome. All Cm’ clones were tested by PCR using chromosomal DNA as a

### TABLE 1. B. subtilis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>PY79</td>
<td>Parental strain</td>
<td>33</td>
</tr>
<tr>
<td>DB068</td>
<td>cotB::cat</td>
<td>8</td>
</tr>
<tr>
<td>ER203</td>
<td>cotB::erm</td>
<td>27</td>
</tr>
<tr>
<td>RH103</td>
<td>amyE::cotB-tetC fusion A (Fig. 1B)</td>
<td>This study</td>
</tr>
<tr>
<td>RH109</td>
<td>amyE::cotB-tetC fusion C (Fig. 1B)</td>
<td>This study</td>
</tr>
<tr>
<td>RH110</td>
<td>amyE::cotB-tetC fusion B (Fig. 1B)</td>
<td>This study</td>
</tr>
<tr>
<td>RH201</td>
<td>cotB::spe</td>
<td></td>
</tr>
<tr>
<td>RH205</td>
<td>cotB::spe amyE::cotB-tetC fusion A (Fig. 1B)</td>
<td>This study</td>
</tr>
<tr>
<td>RH206</td>
<td>cotB::spe amyE::cotB-tetC fusion C (Fig. 1B)</td>
<td>This study</td>
</tr>
<tr>
<td>RH207</td>
<td>cotB::spe amyE::cotB-tetC fusion B (Fig. 1B)</td>
<td>This study</td>
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</table>

FIG. 1. (A) CotB amino acid sequence (16; accession number P07789). The three 27-amino-acids repeats are underlined. The arrow indicates the last CotB amino acid residue (at position 275) in fusion A and fusion B (see below); the 41 amino acid residues used in fusion C are double underlined (see below). (B) cotB gene and strategy for construction of CotB-TTFC gene fusions. tetC (1,137 bp) was cloned in frame to either the 3′ (fusions A) or the 5′ (fusion B) end of the 825-bp DNA fragment coding for the first 275 amino acids of CotB. Fusion C was derived from fusion A by cloning of a DNA fragment (123 bp) coding for the last 41 amino acids of CotB (double underlined in panel A) in frame with the 3′ end of TTFC.
of pUC19 derivative carrying the RH201. RH201 was obtained by transforming strain PY79 with plasmid pRH30, DNA (Fig. 1B), thus indicating the occurrence of correct recombination events. Amplification of 4,057 bp, while clones derived from plasmid pNS9 showed a slightly larger fragment. The inoculum of 10^10 spores from strain RH103, carrying fusion A, and the wild-type spore. Extracted proteins were fractionated on 10 or 12.5% denaturing polyacrylamide gels, electrotransferred to nitrocellulose filters (Bio-Rad), and used for Western blot analysis. The number of purified spores obtained was measured by direct counting with a Bürker chamber under an optical microscope (Olympus BH-2 with 40× lenses).

Aliquots of 10^11 spores suspended in 0.30 ml of distilled water were used to extract coat proteins by sodium dodecyl sulfate (SDS)-dithiothreitol (DTT) treatment as previously described (22). The number of purified spores obtained was measured by direct counting with a Bürker chamber under an optical microscope (Olympus BH-2 with 40× lenses).

A total of 10^5 purified spores were washed in phosphate-buffered saline (PBS) and incubated for 1 h at 37°C with specific antibodies. The presence of TTFC-specific IgG antibodies was assayed by enzyme-linked immunosorbent assay as previously described (24). Flat-bottom microtiters plates (high-binding capacity; Greiner, Frickenhausen, Germany) were coated with 100 μl of recombinant TTFC per well (1 μg/ml; Boehringer Mannheim), and serum samples initially diluted 1:20 and titrated in twofold dilutions in duplicate were added. After a 2-h incubation at 37°C, the plates were washed and alkaline phosphatase-conjugate goat anti-mouse IgG (I:1,500, Southern Biotechnologies Associates, Birmingham, Ala.) was added. The alkaline phosphatase substrate was added, and the plates were read after 1 h at 405 nm by using a 340 ATC reader (SLT Labinstrument). The TTFC-specific IgG concentration in each sample was calculated using a standard curve of anti-TTFC monoclonal antibody (Boehringer GmbH, Mannheim, Germany), starting from a concentration of 8 ng/ml and titrated in twofold dilutions in duplicate.

Samples were tested individually, and data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined by Student’s unpaired t test, and the significance level was set at P < 0.05.

RESULTS

CotB is expressed on the surface of B. subtilis spores. At least 20 polypeptides are organized to form the two layers of the B. subtilis spore coat (9, 14). Some of these polypeptides have been associated with the outer part of the coat, but for all of them the exact location within the coat and the protein domain required for surface exposure are not known. To identify an externally exposed coat component, we investigated the localization of CotB, a 42.9-kDa component of the B. subtilis spore coat associated with the outer coat layer (34) and characterized by a strongly hydrophilic region at the C-terminal end (17). More than 50% of CotB amino acid residues from residues 248 to 356 are serines (Fig. 1A), and three 27-amino-acid repeats are present from residues 253 to 333 (underlined in Fig. 1A).

Sporules were purified from a B. subtilis wild-type strain (PY79) (33) and an isogenic mutant strain carrying a Cam′ gene cassette (cat) interrupting the cotB coding region (BD067) (8). Purified spores were reacted with CotB-specific antibodies and run in a cytofluorimetric assay (Fig. 2). Specific fluorescent staining of the spore surface was observed only with PY79 spores (Fig. 2), indicating that in wild-type spores CotB was accessible to antibodies and thus probably externally exposed.

Based on these results, CotB was selected as a fusion partner to attempt the expression of heterologous polypeptides on the spore surface.

As a model system, we analyzed the surface display of the 459-amino-acid TTFC, a well-characterized (13) and highly immunogenic (20) 51.8-kDa peptide, encoded by the tetC gene of Clostridium tetani, which is easily detectable since both monoclonal and polyclonal anti-TTFC antibodies are available.

<table>
<thead>
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<th>Table 2. cotB synthetic oligonucleotides</th>
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<tr>
<td>Oligonucleotide</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>B1</td>
</tr>
<tr>
<td>B3</td>
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<tr>
<td>B4</td>
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<tr>
<td>B5</td>
</tr>
<tr>
<td>B6</td>
</tr>
<tr>
<td>B7</td>
</tr>
</tbody>
</table>

* Capital and lowercase letters indicate bases of cotB DNA and of an unpaired tail carrying a restriction site (the restriction site is underlined), respectively.
  * Position of annealing refers to the cotB sequence, with the first position of the translational initiation codon as +1 (9).
Construction and chromosomal integration of the CotB-TTFC gene fusion. The strategy to obtain recombinant *B. subtilis* spores expressing TTFC on their surface was based on (i) use of the cotB gene and of its promoter for the construction of translational fusions and (ii) chromosomal integration of the cotB-tetC gene fusions into the coding sequence of the nonessential gene *amyE* (5).

To avoid potential stability problems of the genetic constructs, cotB DNA encoding the three 27-amino-acid repeats of CotB (Fig. 1A) was not included in the gene fusions and only DNA encoding the N-terminal 275 amino acids of CotB was used (Fig. 1B). Fusion A (Fig. 1B) was obtained by cloning tetC in frame with the serine codon at position 275 (Fig. 1A); fusion B (Fig. 1B) was constructed by inserting tetC between cotB transcriptional-translational signals and cotB DNA coding for the first 275 amino acids; fusion C (Fig. 1B) was derived from fusion A by addition of DNA coding for the C-terminal 41 amino acids of CotB (Fig. 1A).

All gene fusions were integrated on the *B. subtilis* chromosome at the *amyE* locus by double-crossover recombination events (see Materials and Methods). Individual clones for each transformation were tested by PCR (data not shown); these clones were named RH103 (fusion A), RH110 (fusion B), and RH109 (fusion C) and were used for further analysis.

The three recombinant strains and their isogenic parental strain PY79 showed comparable sporulation and germination efficiencies, and their spores were equally resistant to chloroform and lysozyme treatment (data not shown), indicating that the presence of the CotB-TTFC fusion does not significantly affect spor structure and/or function.

**CotB-TTFC gene fusions are localized to the spore coat.** To verify that CotB-TTFC gene fusions were localized to the spore coat, we analyzed cell extracts of sporulating cells in Western blot experiments with anti-CotB and anti-TTFC antibodies. Strains RH103 (fusion A), RH110 (fusion B), and RH109 (fusion C) were grown in Difco sporulation (DS) medium and harvested 7 h after the onset of sporulation, at the time of maximal cotB expression (9, 14). Cells were disrupted by sonication, and the total-cell extract (supernatant) was separated from forming spores, unbroken cells, and cell debris (pellet) by centrifugation. The obtained pellet was then used to extract coat proteins by SDS-DTT treatment at 65°C. Then 50-µg portions of total proteins obtained from supernatant and pellet of each strain were fractionated on a 10% polyacrylamide gel, blotted, and reacted with anti-CotB and anti-TTFC antibodies. As shown in Fig. 3 (lanes 1 to 8), specific CotB-TTFC signals were observed only in the pellet fractions of the three strains. Our interpretation of these results was that CotB-TTFC was assembled on the forming spore (pellet) and,

![FIG. 2. Flow cytometric analysis of the spore surface.](image_url)

*B. subtilis* spores were prepared from wild-type strain PY79 (A) and the isogenic cotB mutant BD067 (B), and the analysis was performed on the entire spore population (ungated). Open histogram: spores reacted with CotB-specific rabbit polyclonal antibodies, and with FITC-conjugated secondary antibodies. Filled histogram: spores reacted only with FITC-conjugated secondary antibodies. a.u. indicates arbitrary units of fluorescence intensity. A total of 7,400 positive events (spores whose fluorescence was significantly higher than the unspecific fluorescence due to the spore without the addition of primary and secondary antibodies) of 10,000 spores analyzed were scored for the open histogram of panel A.

**FIG. 3.** Western blot of proteins extracted from sporulating cells of strains carrying the TTFC fusions in a wild-type (lanes 1 to 8) and an isogenic cotG null (lanes 9 to 16) background. Collected cells were sonicated, and forming spores (pellet [lanes 1 to 4 and 9 to 12]) were separated from cell extracts (supernatant [lanes 5 to 8 and 13 to 16]). Lanes: 1 and 5, wild-type strain PY79; 9 and 13; cotG null strain ER203; 2, 6, 10, and 14, fusion A; 3, 7, 11, and 15, fusion B; 4, 8, 12, and 16, fusion C. Total proteins (50-µg portions) were fractionated on a 10% polyacrylamide gel and, on electrotransfer to nitrocellulose membranes, were reacted with TTFC-specific rabbit antibodies and then with secondary antibodies and visualized by the enhanced chemiluminescence method. Molecular mass markers (in kilodaltons) are indicated. Identical results were obtained with anti-CotB antibodies (data not shown).
as a consequence, not found in the cell extracts (supernatant). To determine whether this interpretation was correct, we used chromosomal DNA-mediated transformation to move the gene fusions into an isogenic *B. subtilis* strain with the cotG gene deleted (ER203; Table 1), since CotG is known to be required for CotB assembly within the spore coat (27). We then repeated the experiment as described above and collected sporulating cells 7 h after the onset of sporulation. As shown in Fig. 3 (lanes 9 to 16), in a cotG null background CotB-TTFC specific signals were found in the cell extract fraction (supernatant), thus allowing us to infer that CotB-TTFC gene fusions are spore coat associated.

**Surface display of TTFC on the recombinant spores.** Western blot analysis of spore coat proteins purified from recombinant strains revealed the presence of a ca. 82-kDa band which reacted with both TTFC- and CotB-specific rabbit antibodies (Fig. 4A). A 59-kDa band only reacting with CotB-specific antibodies was present in extracts from wild type and recombinant spores (Fig. 4A), indicating the presence of intact CotB in the wild-type (lane 1) or an isogenic cotB (lane 2) background; fusion B in the wild-type (lane 3) or an isogenic cotB (lane 4) background; or fusion C in the wild-type (lane 5) or an isogenic cotB (lane 6) background. Purified TTFC was run in lane 7. Extracted proteins (15-μg portions) were fractionated on a 12.5% polyacrylamide gel and, on electrotransfer to nitrocellulose membranes, were reacted with TTFC-specific rabbit antibodies and then with secondary antibodies and visualised as described for panel A. Molecular mass markers (in kilodaltons) are indicated.

To analyze whether the intact copy of CotB was needed for surface expression of CotB-TTFC, we used chromosomal DNA-mediated transformation (5) to move the gene fusions into a cotB mutant strain (RH201; Table 1). Western blot experiments, performed with coat proteins from strains carrying fusion A in the wild-type (lane 1) or an isogenic cotB (lane 2) background; fusion B in the wild-type (lane 3) or an isogenic cotB (lane 4) background; or fusion C in the wild-type (lane 5) or an isogenic cotB (lane 6) background. Purified TTFC was run in lane 7. Extracted proteins (15-μg portions) were fractionated on a 12.5% polyacrylamide gel and, on electrotransfer to nitrocellulose membranes, were reacted with TTFC-specific rabbit antibodies and then with secondary antibodies and visualised as described for panel A. Molecular mass markers (in kilodaltons) are indicated.

A quantitative determination of the amount of TTFC exposed on *B. subtilis* spores was obtained by dot blot experiments using serial dilutions of purified TTFC and of coat proteins extracted from spores of the wild-type and recombinant strains. Proteins were reacted with monoclonal anti-TTFC antibody and then with alkaline phosphatase-conjugated secondary antibodies, and the color was developed by the BCIP/NBT system (Bio-Rad). Figure 6 shows the results obtained with strain RH103, carrying fusion A. Identical results were obtained with strains RH109 and RH110, carrying...
fusion C and fusion B, respectively (data not shown). A densitometric analysis indicated that the CotB-TTFC fusion protein amounted to 0.7% of the total coat proteins extracted (Table 3). Since under our experimental conditions an average of 0.032 (±2%) pg of total coat proteins was reproducibly extracted from each spore of the recombinant strains (see Materials and Methods), we calculated that 0.00022 pg of CotB-TTFC fusion protein was extracted from each spore. Based on that and on the deduced molecular mass of 83.1 kDa for the CotB-TTFC fusion protein, we estimated that ca. 1.5 × 10^3 recombinant molecules were extracted from each spore.

**DISCUSSION**

Some features of spore-forming bacilli, such as fast growth, relatively simple nutritional requirements, and efficient secretion of large amounts of proteins and other metabolites, make these organisms particularly suitable for industrial fermentations. Strains of species like *B. licheniformis* and *B. amyloliquefaciens* have been used for the production of various proteins of homologous origin, such as proteases, lipases, and starch-degrading enzymes. Some species, especially *B. subtilis*, have also been used as hosts for the production of heterologous proteins (1, 16, 18). Genetic tools for efficient expression and secretion of recombinant proteins in the latter organism have been rapidly developed and successfully used (10, 23).

In contrast to these extensive applications of vegetative cells of spore-forming bacilli, it is surprising that little attention has been devoted to the spore as a vehicle of recombinant proteins, since the high stability of the spore provides obvious additional advantages. The study reported here is to our knowledge the first to describe the engineering of a spore and to suggest that

![Flow cytometric analysis of TTFC expression on the spore surface.](image)

**FIG. 5.** Flow cytometric analysis of TTFC expression on the spore surface. *B. subtilis* spores were purified from strains carrying fusion A (A), fusion B (B), or fusion C (C) and from an isogenic wild-type strain (D), and the analysis was performed on the whole spore population (ungated). Open histograms: spores reacted with TTFC-specific rabbit polyclonal antibodies and with FITC-conjugated secondary antibodies. Filled histograms: spores reacted only with FITC-conjugated secondary antibodies. a.u. indicates arbitrary units of fluorescence intensity. A total of 3,200, 3,100, and 2,900 positive events (spores whose fluorescence was significantly higher than the nonspecific fluorescence due to the spore without the addition of primary and secondary antibodies) out of 10,000 spores analyzed were scored for the open histograms of panels A, B, and C, respectively.
recombinant *B. subtilis* spores with relevant features such as safety, stability, easy preparation, and low cost may become very attractive vectors of biologically active molecules.

Detailed genetic and morphological studies have shown that the *B. subtilis* spore is surrounded by a multifaceted coat (9, 14), whose proteinaceous nature immediately suggests the potential possibility of using its structural components as fusion partners for the expression of heterologous proteins on the spore surface. In this study we analyzed this possibility by using as fusion partner CotB, a 380-amino-acid coat protein with a strongly hydrophilic C-terminal end containing three 27-amino-acid repeats (residues 252 to 333) (17). We show here that CotB is externally exposed and can be successfully used to express and expose on the spore surface the highly immunogenic TTFC.

**TABLE 3.** Densitometric analysis of dot blot experiments

<table>
<thead>
<tr>
<th>TTFC source</th>
<th>Amt of protein used</th>
<th>Density (OD/mm²)</th>
<th>TTFC concentration conc (ng) in extracts (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified TTFC</td>
<td>6.25 ng</td>
<td>5.19 (±0.07)</td>
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</tr>
<tr>
<td></td>
<td>3.12 ng</td>
<td>3.30 (±0.05)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>1.56 ng</td>
<td>1.62 (±0.02)</td>
<td>NA</td>
</tr>
<tr>
<td>Coat protein extract</td>
<td>0.93 mg</td>
<td>5.35 (±0.07)</td>
<td>6.44 (0.69)</td>
</tr>
<tr>
<td></td>
<td>0.46 mg</td>
<td>3.58 (±0.05)</td>
<td>3.38 (0.73)</td>
</tr>
<tr>
<td></td>
<td>0.23 mg</td>
<td>1.64 (±0.02)</td>
<td>1.58 (0.68)</td>
</tr>
</tbody>
</table>

*NA, not applicable.*

The low fluorescence levels observed when the recombinant spores were reacted with specific anti-TTFC antibodies (Fig. 5) were probably due to the presence on the spore surface of CotB together with CotB-TTFC (Fig. 4). The observed fluorescence was, however, highly specific (no background was obtained with wild-type spores) (Fig. 5D) and very unlikely to be due to broken or defective spores, since sample alterations in cytofluorimetric assays usually result in the appearance of diverse cell types that were not observed in our un gated analysis and since comparable numbers of fluorescent spores were constantly detected in several experiments with all three recombinant strains.

To avoid potential structural instability of the genetic constructs, the three 27-amino-acid repeats of CotB were not included in the fusions with TTFC. The CotB-TTFC coding DNA was put under *cotB* transcriptional and translational signals and integrated onto the *B. subtilis* chromosome to ensure correct timing of expression during sporulation and high stability of the genetic construct, respectively.

Unaltered resistance of recombinant spores to chloroform and lysozyme suggested that the presence of TTFC does not affect the structure and/or function of the spore coat. This is not surprising, since most structural coat proteins, including CotA, CotB, CotC, CotD, and CotF, are functionally redundant and the absence of any one of them does not cause evident phenotypic alterations (9, 14).

Surface display of the CotB-TTFC recombinant proteins on the spore appeared similarly efficient for all three fusions, with estimated yields of 1.5 × 10⁴ CotB-TTFC molecules per spore, and was dependent on the presence of an intact copy of CotB. Such dependence is an interesting result that points to a possible interaction among CotB molecules within the coat and that deserves to be specifically addressed in a separate context.

Immunogenicity of the recombinant spores in mice suggested that TTFC exposed on the spore surface is in a biologically active form. Although for ethical reasons we performed the immunization experiments with spores of only one recombinant strain (RH103), the identical results obtained with the three fusions in Western, dot blot, and cytofluorimetric analyses suggest that TTFC exposed on the surface of spores of the other two strains would be similarly immunogenic in mice.

The size of the heterologous protein to be expressed is often a critical point of surface display systems. The high levels of expression we observed with TTFC, a large peptide of 459 amino acids and a deduced molecular mass of 51.8 kDa, suggest that our spore-based display strategy may have less size restrictions than other cell- or phage-based systems.

**ACKNOWLEDGMENTS**

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