Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system

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Surface display systems are a powerful biological tool with a variety of applications in the development of live vaccines, treatment of microbial infections, screening of peptide libraries, and generation of biocatalysts or biosensors (1,2). Several approaches to display heterologous proteins on bacteria and phages have been developed and extensively reviewed (2,3). More recent is a strategy to engineer the *Bacillus subtilis* endospore (spore) to display heterologous proteins on its surface (4). In comparison to systems based on the use of phages or bacterial cells, the spore-display system provides several advantages, including high stability and safety. Spores are extremely stable life forms generated by gram-positive bacteria of the *Bacillus* and *Clostridium* genera in response to harsh environmental conditions that do not allow cell growth and survival (5,6). The commercial use of spores of various species of the *Bacillus* genus as probiotics or for the oral prophylaxis of gastrointestinal disorders clearly proves the safety of spores of those species (7).

The spore-display system, thus far used to express antigens (4,8,9) and enzymes (10), is based on the construction of gene fusions between heterologous DNA and a *B. subtilis* gene coding for a component of the coat, the proteinaceous structure surrounding the spore (5). CotC is a 66-amino acid component of the *B. subtilis* spore coat, which was previously used to display two heterologous proteins: the C fragment of the tetanus toxin (TTFC) and the B subunit of the heat labile toxin (LTB) of *Escherichia coli* (8). Mauriello et al. (8) showed that the amount of CotC-TTFC exposed on the surface of each recombinant spore was comparable to that previously detected with spores expressing TTFC fused to another coat protein, CotB (4). This was unexpected since CotC is more abundant than CotB in a mature spore coat (5). Based on this observation, we hypothesized that the use of CotC as a fusion partner could be improved. Increased efficiency of this display system is highly desirable for practical applications, such as the delivery of the required dose of antigen by the lowest number of spores or the development of high performance biocatalyzers and biosensors.

To explore the possibility of improving the display efficiency of CotC-based chimeras, a short DNA fragment coding for a 6×His-tag was fused in-frame to either the 3′ or 5′ end of the *cotC* gene. Both fusions were integrated on the chromosome of strain RH209. Strain RH209 carries null mutations in both *cotC* and *cotU*, a *cotC*-homolog whose product is recognized by Western blot analysis with anti-CotC antibody (11). Purified spores were used to extract coat proteins as previously reported (12), and Western blot analysis was performed with anti-CotC. When CotC was tagged at its N-terminal end, we observed a wild-type CotC pattern with a monomer and a homodimer (11). However, when CotC was tagged at its C-terminal end, only a reduced amount of the CotC monomer was found in the spore coat (data not shown). Those results, therefore, indicate that the C-terminal end of CotC has to be free to allow efficient assembly and homodimerization of the protein, suggesting the N-terminal end of CotC as a better site for the insertion of heterologous proteins to be displayed.

Table 1. Synthetic Oligonucleotides

Oligonucleotide	Sequence[a]	Position of Annealing[b]
TTFCsenso	5′-CGGCCG**TCAACACCAATTCCATTTT**-3′	+340/+359
TTFCanti	5′-CGCCGGCG**AATCATTTGTCCTCCATC**-3′	+1705/+1720
GFP-*Not*IFW	5′-GCGGCCGC**TAAGGAGAAGAACTTTTCA**-3′	+3/+19
GFP-*Not*IR	5′-AAAGCGGCCGCT**TTTGTATAGTTCATCCATGCC**-3′	+693/+714
CotCp	5′-ACATGCATGC**TGTAGGATAAATCGTTTG**-3′	-179/-161
CotCstop	5′-GTCGACTTA**TTAGTAGTGTTTTTTATGC**-3′	+138/+201
CpoI1	5′-GCCGCTAGCATCGATCGGCCGC**ATATACTCCTCC**-3′	+3/-12
CpoI2	5′-CCGATCGATGCTAGCGGCCGC**ATGGGTTATTACAAAAAATAC**-3′	+1/+21

[a]Uppercase letters indicate cleavage sites for restriction enzymes, bolded uppercase letters indicate bases of complementarity with genes coding for the C fragment of the tetanus toxin (TTFC), green fluorescent protein (GFP), CotC, or AmyE. Underlined letters indicate the overlapping region between the two internal primers.
[b]Refers to genes encoding for TTFC, GFP, CotC, or AmyE, considering the first nucleotide of the translational start site is +1.

Transcriptional and translational signals of the gene coding for the fusion partner and an integrative vector for chromosomal integration of the fusions are needed to obtain efficient and stable expression of heterologous proteins (4,8). To simplify the cloning of heterologous DNA between *cotC* regulatory and coding regions, we inserted a DNA fragment carrying three restriction sites downstream of the *cotC* promoter and ribosome-binding site and upstream of the *cotC* coding region, in-frame with the *cotC* translational start site (Figure 1A). To this aim, we used the gene splicing by overlap extension (SOEing) technique (13) with the *B. subtilis* chromosome as template and synthetic oligonucleotides CotCp, Cpol1, CotCstop, and Cpol2 as primers (Table 1). The modified version of *cotC* was then cloned into the integrative plasmid pDG364 (14), yielding plasmid pDS17, an *E. coli-B. subtilis* shuttle plasmid that allows, by a double crossing-over event, the replacement of the unessential *amyE* gene on the *B. subtilis* chromosome with plasmid DNA (Figure 1A).

To validate the use of the vector, the *gfp* gene, encoding the green fluorescent protein (GFP) (15), was amplified using plasmid pAD123 (*Bacillus* Genetic Stock Center; BGSC, www.bgsc.org) as GFP template and oligonucleotides GFP-*Not*IFW and GFP-*Not*IR as primers (Table 1) and cloned into plasmid pDS17. The recombinant plasmid was then integrated into chromosome of the *B. subtilis* strain RH209, yielding strain DS127.

Coat proteins were extracted from purified spores of strains DS127 and RH209 as previously described (12), and Western blot analysis was performed with anti-CotC antibody. Since both strains have a null mutation in the *cotC* gene (and in its structural homolog *cotU*), the anti-CotC antibodies were expected to recognize only the CotC-based chimera. As predicted, no proteins were recognized in RH209; while in DS127, spores two proteins of approximately 40 and 80 kDa were recognized by the anti-CotC antibody (Figure 1B). Since GFP is a 27-kDa protein (15), and the monomeric form of CotC migrates on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as 12 kDa (11), the size of the 40-kDa protein conforms well with the size expected for a CotC-GFP chimera. The 80-kDa protein, also recognized by anti-CotC antibody, would then be a CotC-GFP dimer. CotC-GFP spores were also analyzed by fluorescence microscopy. Figure 1, C and D, show the same field observed by phase contrast (Figure 1C) or fluorescence microscopy (Figure 1D). Fluorescence was not associated with vegetative cells, but was specific to the spore form and concentrated around the spore, as previously reported for other coat components (16).

To evaluate the display efficiency of our new system, we placed TTFC at the N-terminal end of CotC, previously inserted at the C terminus of the fusion partner (8). *Clostridium tetani* DNA encoding TTFC was amplified using the *B. subtilis* chromosome of strain RH103 (4) as TTFC template

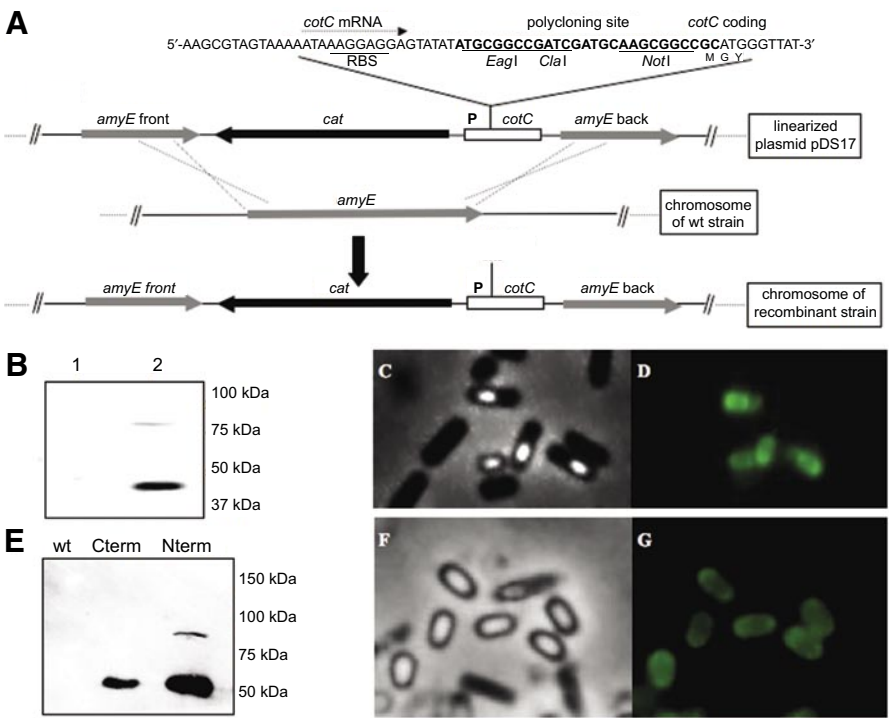

Figure 1. (A) Representation of plasmid pDS17 and of the chromosome integration event. The nucleotide sequence of the polycloning site is reported in bold. Underlined letters are the recognition sequence for the restriction enzymes indicated below and for ribosomes (indicated as RBS). (B) Western blot analysis. Coat proteins extracted from 24-h-old spores of strain RH209 (*cotU::neo cotC::spc*) (lane 1) and of the congenic strains DS127 (*cotC::gfp*) (lane 2). Proteins were fractionated on 10% polyacrylamide gel, and upon electrotransfer on nitrocellulose membranes, reacted with CotC-specific rabbit antibodies, then with peroxidase-conjugated secondary antibodies, and visualized using the Pierce method. Molecular mass of a marker (Precision Plus Protein Standards; Bio-Rad Laboratories, Hercules, CA, USA) is indicated. (C and D) Localization of CotC-green fluorescent protein (GFP). The same microscope field is shown by (C) phase contrast and by (D) fluorescence microscopy. Cells of strain DS127 were induced to sporulate, collected 6 h after the onset of sporulation, washed, and spotted onto microscope slides, and firmly covered with 0.1% poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO, USA)-treated coverslips as described in Reference 16. (E) Western blot analysis with anti-CotC antibody of coat proteins extracted from 24-h-old spores of the congenic strains RH209 (*cotU::neo cotC::spc,* indicated as wt), DS125 (*cotU::neo cotC::spc cotC-tetC*-Cterm), and DS124 (*cotU::neo cotC::spc cotC-tetC*-Nterm). Western blot analysis was performed as described for panel B, with the only difference being that proteins were fractionated on 7% polyacrylamide gel. (F and G) Immunofluorescence microscopy localization of CotC-TTFC. The same microscope field is shown by (F) phase contrast and by (G) immunofluorescence microscopy. Samples were reacted with CotC-specific rabbit antibodies, then with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Immunofluorescence microscopy was performed as described in Reference 17. Fluorescence and immunofluorescence microscopy was performed with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a 100× UPlanF1 objective and a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter 515°–550° nm). Exposure times were typically in the range of 500–1000 ms. Images were captured and cropped with analySIS software (SIS; Software Imaging Systems, Munster, Germany). TTFC, C fragment of the tetanus toxin.

and oligonucleotides TTFCsenso-TTFCanti as primers (Table 1) and was cloned into plasmid pDS17. The recombinant plasmid was then integrated into the chromosome of the *B. subtilis* strain RH209, yielding strain DS124. Coat proteins were extracted from purified spores of strains RH209, DS124 (CotC-TTFC-Nterm), and DS125 (CotC-TTFC-Cterm) as previously described (12), and Western blot analysis was performed with anti-CotC antibody. A clear improvement of TTFC display was observed with strain DS124 with respect to strain DS125. While two specific signals of approximately 60 and 120 kDa were observed with DS124 spores, a single, less abundant protein of approximately 60 kDa was found with DS125 spores (Figure 1E). Since TTFC is a 51-kDa protein (4,8), the 60-kDa protein conforms well with the expected size for a CotC-TTFC chimera. The 120-kDa protein, also recognized by anti-CotC antibody, would then be a CotC-TTFC dimer.

A quantitative determination of the amount of TTFC exposed on DS124 and DS125 spores was obtained by dot blot experiments. Densitometric analysis of the dot blot experiment indicated that while CotC-TTFC (Cterm) represented approximately 0.3% of the extracted coat proteins, CotC-TTFC (Nterm) was about 5-fold more abundant (Table 2). Based on previously reported data, estimating that approximately 9.7×10^2 CotC-TTFC molecules were extracted from each spore of strain DS125 (8), we calculated that approximately 4.8×10^3 CotC-TTFC molecules were extracted from each spore of strain DS124.

CotC-TTFC spores were also analyzed by immunofluorescence microscopy. Figure 1, F and G, show the same field observed by phase contrast (Figure 1F) or fluorescence microscopy (Figure 1G). As in the case of CotC-GFP (Figure 1, C and D), fluorescence was exclusively concentrated around the spore. The accessibility of the CotC-TTFC chimera by primary and secondary antibodies in the immunofluorescence microscopy experiments also indicated that the CotC-TTFC chimera is exposed on the spore surface. Recombinant spores expressing GFP or TTFC did not differ from congenic wild-type spores in the efficiency of sporulation, spore resistance to heat and chemicals, and efficiency of germination (data not shown).

Table 2. Densitometric Analysis of the Dot-Blot Experiment

	Amount of Protein Used (ng)	Density (A/mm²)[a]	CotC-TTFC in Extract (% total ng)[b]
Purified CotC	25	8.19 (±0.03)	N.A.
	12.5	3.35 (±0.02)	N.A.
	6.25	1.89 (±0.01)	N.A.
Coat Proteins Extracted from Strain DS125 (Cterm)	5000	4.04 (±0.02)	14.70 (0.29)
	2500	1.87 (±0.01)	7.51 (0.30)
	1250	0.91 (±0.01)	3.72 (0.29)
Coat Proteins Extracted from Strain DS124 (Nterm)	2500	9.52 (±0.03)	40.20 (1.60)
	1250	4.99 (±0.02)	21.00 (1.53)
	625	2.57 (±0.01)	9.30 (1.49)

[a]Density was measured as absorbance (A) per square millimeter and obtained by GelDoc™ 2000 apparatus with Multi-Analyst™ software (both from Bio-Rad Laboratories, Hercules, CA, USA).
[b]Results were calculated from signals (density A/mm²) obtained with known concentrations of purified CotC.
N.A., not applicable.

The spore-display system presented here is 5-fold more efficient than the previous procedure (8) and, using a *B. subtilis* strain that does not contain other *cotC* alleles, allows the unambiguous immune detection of the exposed molecule without the need of raising specific antibody against each displayed protein. The system has been validated by using a short peptide (6×His-tag) and two proteins, the 27-kDa GFP and the 51-kDa TTFC. The high levels of expression we observed with those large proteins suggest that our spore-based display strategy may become a useful tool for drug and vaccine delivery and for the development of new bioreactors and biosensors.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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