ABSTRACT A variety of bioactive peptides and proteins have been successfully displayed on the surface of recombinant spores of *Bacillus subtilis* and other sporeformers. In most cases, spore display has been achieved by stably anchoring the foreign molecules to endogenous surface proteins or parts of them. Recombinant spores have been proposed for a large number of potential applications ranging from oral vaccine vehicles to bioremediation tools, and including biocatalysts, probiotics for animal or human use, as well as the generation and screening of mutagenesis libraries. In addition, a nonrecombinant approach has been recently developed to adsorb antigens and enzymes on the spore surface. This nonrecombinant approach appears particularly well suited for applications involving the delivery of active molecules to human or animal mucosal surfaces. Both the recombinant and nonrecombinant spore display systems have a number of advantages over cell- or phage-based systems. The stability, safety of spores of several bacterial species, and amenability to laboratory manipulations, together with the lack of some constraints limiting the use of other systems, make the spore a highly efficient platform to display heterologous proteins.

SURFACE DISPLAY ON BACTERIAL CELLS OR PHAGES

Display systems that present biologically active molecules on the surface of microorganisms have become increasingly used to address environmental and biomedical issues (1–3). Strategies using environmentally relevant proteins or peptides for display on the surface of phages or bacterial cells have been extensively reviewed by Wu et al. (4). Examples include proteins able to bind metal ions that can be used as biosorbents or biocatalysts, including cysteine-rich metallothioneins (MTs) or Cys-His rich synthetic peptides, known to bind Cd$^{2+}$ and Hg$^{2+}$ with a very high affinity. Eukaryotic MTs have been expressed on the surface of *Escherichia coli* cells through fusion to the porin LamB, with a 20-fold increased ability of Cd$^{2+}$ accumulation of the recombinant cell with respect to its parental strain (5, 6).

In addition, metal-binding peptides have also been expressed on the surface of soil bacteria known to survive in contaminated environments. The mouse MT was displayed on the surface of *Pseudomonas putida* (7) and *Ralstonia metallidurans* CH34 (8), resulting in a 3-fold increase in binding and removal of Cd$^{2+}$, sufficient to improve plant growth in a contaminated soil (8). Synthetic phytochelatins (ECn) with the repetitive metal-binding motif (Glu-Cys)nGly were displayed on the surface of *Moraxella* sp. cells causing a 10-fold improvement in Hg$^{2+}$ intracellular accumulation (9–11). In addition to heavy metals, organic contaminants can be removed from the environment by the use of microbial cells displaying heterologous enzymes. Examples include organophosphorus hydrolases (OPHs). These bacterial enzymes are able to degrade organophosphates, which are toxic compounds widely used as pesticides. *E. coli* cells expressing OPH on their surface via the Lpp-OmpA fusion system were able to degrade parathion and paraoxon 7-fold faster than cells expressing OPH intracellularly (12). Surface display approaches have also been used to develop whole-cell diagnostic tools and vaccine delivery systems. Functional single-chain antibody fragments have been expressed on bacterial cells and used as diagnostic devices in immunological tests. In the first report of an antibody fragment expressed in an active form on a bacterial surface, the murine anti-human-IgE scFv antibody fragment was exposed on the surface of...
Staphylococcus xylosus and S. carnosus cells (13). More recently, the oral commensal bacterium Streptococcus gordonii was engineered to display a single-chain Fv (scFv) antibody fragment, derived from a monoclonal antibody raised against the major adhesin of the dental caries-producing bacterium Streptococcus mutans (streptococcal antigen I/II or SA I/II). Recombinant S. gordonii was found to specifically bind to immobilized SA I/II and represents the first step toward the development of a stable system for the delivery of recombinant antibodies (14).

Surface Display for Vaccine Delivery
The most common application of surface display systems has been the development of new vaccines. While the display of peptides on phages has been used mostly as a tool for epitope discovery, the display on bacterial surfaces usually takes advantage of previously identified vaccine candidates (immunogenic peptides or proteins) to obtain a recombinant bacterium that can then be used as a live carrier for vaccine delivery (15). The possibility of using phage display for the identification of neutralizing viral epitopes was first demonstrated by inserting peptide sequences from the V3 loop of gp120 from HIV-1 into the N-terminal region of the major coat protein (pVIII) of the filamentous bacteriophage fd, which led to their display in multiple copies on the surface of the virion (16). While most of the initial studies were carried out using filamentous phage fd, other phage systems were later developed for similar purposes (17–20). Initial attempts at using bacterial display for the development of new vaccines were focused on Gram-positive bacteria (21, 22) and E. coli, Salmonella, and other Gram-negative bacteria were considered only later (23). Studies on Salmonella provided clear evidence that attenuated strains can be effectively used as a general vaccine vehicle to deliver antiphagocytic virulence determinants of unrelated bacteria (24, 25). To avoid the use of engineered pathogens for vaccine delivery, systems that allow the expression of heterologous antigens on the surface of commensal bacteria were also developed (26).

Strategies of Surface Display
Strategies to display heterologous proteins on the bacterial surface are generally based on a cell surface molecule (outer membrane proteins, lipoproteins, subunits of cell appendages, S-layer proteins, cell wall and cell membrane proteins) acting as a carrier and able to anchor an heterologous passenger protein on the cell surface (1) (Fig. 1). Several properties of carrier proteins can affect the efficiency of surface display and have different effects on the stability and integrity of the host. A successful carrier protein should have a strong anchoring motif to avoid detachment from the surface and should be resistant to proteases present in the extracellular medium or in the periplasmic space (27). The site of insertion of the heterologous part into the carrier is another important factor, since it can influence the stability, activity, and posttranslational modification of the fusion protein. Therefore, fusions at the N or C terminus of the carrier protein, as well as sandwich fusions (insertions), have been constructed (Fig. 2). In some instances, a linker peptide (spacer) consisting of repeats of 5 to 10 aliphatic amino acid residues is inserted between

![FIGURE 1](https://doi.org/10.1128/microbiolspec.TBS-0011-2012.f1)
the carrier and the passenger to minimize potential steric effects disturbing the correct folding of either protein (Fig. 2). Some characteristics of the passenger protein can also influence the translocation process and efficiency of surface display. The folded structure of the passenger protein, such as the formation of disulfide bridges in the periplasm or the presence of many charged or hydrophobic residues, can affect translocation through the cell membranes (27). In some cases, surface display of heterologous proteins has been taking advantage of the autotransport properties of certain proteins (28). In these cases, the C-terminal end of the autotransporter (the carrier) is anchored at the cell membrane and exposes its own N-terminal end (the passenger) on the external surface of the membrane. Replacement of the passenger domain with a heterologous protein results in the autodisplay of N-terminally fused passengers (3, 29). Autodisplay technology has been used in several biotechnological applications, including the construction of a variety of whole-cell biocatalytic systems (3).

**SURFACE DISPLAY ON BACILLUS SUBTILIS SPORES**

As described in detail elsewhere, *Bacillus* spores are encased in a coat, a protein structure protecting the spore from toxic chemicals, lytic enzymes (30), and phagocytic predation by protozoans (31). The rigidity and compactness of the spore coat immediately suggest the possibility of using its structural components as anchoring motifs for the expression of heterologous polypeptides on the spore surface. A genetic system to engineer the coat of *B. subtilis* spores has been developed and a model passenger has been efficiently displayed (32). The spore-based approach provides several advantages over other display systems, such as high stability even after prolonged storage, the possibility of displaying large multimeric proteins, and safety for human use (see “Advantages of the Spore Surface Display Systems,” below). The spore coat components of *B. subtilis* and other *Bacillus* species that have been used as carriers, the heterologous proteins used as passengers, and their

**FIGURE 2** C-terminal (top), N-terminal (middle), and sandwich (bottom) fusions. Black squares indicate the linker sequence, in some cases used to separate carrier and passenger. doi:10.1128/microbiolspec.TBS-0011-2012.f2
proposed potential applications are listed in Table 1. Because of limited information on the mechanisms of protein incorporation into the coat, the structure of the proteins forming the spore surface, and the existence of anchoring motifs, initial attempts to expose heterologous proteins on the spore surface were focused on two coat components selected for their surface location (CotB; 32) or for their abundance (CotC; 33). Subsequently, additional coat proteins, as well as coat-associated proteins, have been proposed as carriers (Table 1). A review of the various examples of spore surface display is presented in the following subsections organized on the basis of the carrier utilized.

**CotB as a Carrier Protein**

CotB was the first coat protein to be used as a carrier to target a heterologous protein to the spore surface (32). Expression of the cotB gene is under the control of the mother cell-specific sigma factor, σK, and of the transcriptional regulators GerE and GerR (34). CotB has a strongly hydrophilic C-terminal half formed by three serine-rich repeats (Fig. 3). Serine residues account for over 50% of the CotB C-terminal half. CotB has a deduced molecular mass of 46 kDa, but migrates on SDS-PAGE as a 66-kDa polypeptide. The discrepancy between measured and deduced molecular mass has been explained by showing that CotB is initially synthesized as a 46-kDa species (CotB-46) and converted into a 66-kDa species (CotB-66), presumably a CotB homodimer (35). Conversion of the CotB-46 form into CotB-66 requires expression of cotG and cotH. In cotG mutants, the CotB-46 species accumulates and undergoes assembly (35). The exact role of CotG in formation of CotB-66 is not known. However, CotG was found to be present in complexes with mainly the CotB-46 form at the time of coat assembly. Moreover,

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<th>Table 1</th>
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CotG and CotB were found to interact directly in a yeast two-hybrid assay (35). Presumably, the interaction of CotG with CotB-46 is essential for the formation of the CotB-66 species. The requirement of \( \text{cotH} \) expression for the assembly of both CotG and CotB-66 is in part explained by the observation that CotG does not accumulate in cells mutant for \( \text{cotH} \). This is reminiscent of the situation with CotC, whose various forms (see below) also do not accumulate in a \( \text{cotH} \) mutant (36). These observations have led to the proposal that CotH, or a CotH-controlled protein, protects CotC and CotG from proteolysis prior to their assembly. CotH or a factor under its control could directly interact with CotC and CotG in a chaperone-like manner, or otherwise with a protease that uses CotC and CotG as a substrate (35–37). The possibility of using CotB as a carrier protein was based on its surface localization, as shown by a fluorescence-activated cell sorter (FACS) analysis with anti-CotB antibody (32). To verify that a heterologous protein fused to CotB could be displayed on the spore surface, two antigens were initially selected as model passenger proteins: (i) the nontoxic 459-amino-acid C-terminal fragment of the tetanus toxin (TTFC), a well-characterized and highly immunogenic peptide of 51.8 kDa encoded by the \( \text{tetC} \) gene of \( \text{Clostridium tetani} \) (38); and (ii) the 103-amino-acid B subunit of the heat-labile toxin (LTB) of enterotoxigenic strains of \( \text{E. coli} \), a 12-kDa peptide encoded by the \( \text{eltB} \) gene (39). The strategy to obtain recombinant \( \text{B. subtilis} \) spores expressing CotB-TTFC or CotB-LTB on their surface was based on (i) use of the \( \text{cotB} \) gene and its promoter for the construction of translational fusions and (ii) chromosomal integration of the \( \text{cotB-tetC} \) and \( \text{cotB-eltB} \) gene fusions into the nonessential gene locus \( \text{amyE} \) (32). Placing the fusion proteins under the control of the \( \text{cotB} \) promoter and ribosome-binding site ensured correct timing of expression during sporulation, while its chromosomal integration guaranteed the genetic stability of the construct. When TTFC and LTB were fused to the C-terminal end of CotB, the chimeric proteins failed to correctly and efficiently assemble on the spore surface (27). In order to bypass such problems, TTFC and LTB were fused to the C-terminal end of a truncated CotB, deleted of 105 C-terminal amino acids (CotB\(_{Δ105}\)), thus removing two of the three amino acid repeats (Fig. 3C). The CotB\(_{Δ105}\)-TTFC chimeric protein was efficiently assembled and exposed on the spore surface (32). A quantitative dot blot showed that each recombinant spore exposed an amount of CotB\(_{Δ105}\)-TTFC fusion protein equal to 0.00022 pg and made it possible to conclude that 1.5 \( \times \) 10\(^3\) chimeric molecules are present on the surface of each recombinant spore (32). Unlike CotB\(_{Δ105}\)-TTFC, CotB\(_{Δ105}\)-LTB was not properly assembled. The strain expressing this chimera showed reduced sporulation and germination efficiencies and its spores were not resistant to lysozyme. These observations, together with the SDS-PAGE analysis of the released coat proteins, suggested that the presence of CotB\(_{Δ105}\)-LTB strongly altered the integrity of the spore coat. An \textit{in silico} analysis (27) showed some identity

**FIGURE 3** (A) Amino acid sequences of the three repeats (R1, R2, R3) present in the C-terminal half of CotB. (B) Hydrophobic (black) and hydrophilic (gray) regions of CotB as deduced by a Kyte-Doolittle plot (ProtScale software on ExPASy). (C) Truncated form of CotB used as a carrier for surface display. doi:10.1128/microbiolspec.TBS-0011-2012.f3
between the chimeric product (in the fusion region) and LytF, a cell wall-associated hydrolase produced by \textit{B. subtilis} during vegetative growth and involved in daughter cell separation \cite{40}. When cells are no longer growing, LytF is degraded by the cell surface protease WprA and the extracellular protease Epr \cite{40}. This raised the possibility that the chimeric product \textit{CotBA}_{105}-LTB could be degraded by those proteases during sporulation \cite{27}. Exposure of \textit{CotBA}_{105}-TTFC on the spore surface required the expression of wild-type alleles of \textit{cotH}, \textit{cotG}, and \textit{cotB} \cite{32}. As discussed above, these three gene products act in a hierarchical way with CotH to assemble CotG which, in turn, interacts with CotB. This interaction is essential for the formation of the CotB homodimer of 66 kDa \cite{35}. This would suggest that a wild-type copy of CotB forms a heterodimer with a copy of \textit{CotBA}_{105}-TTFC, allowing the surface display of the fusion. The model passenger protein TTFC has been also fused at the N-terminal and inserted in the middle of CotB \cite{32} (Fig. 2). In both cases the \textit{CotBA}_{105} form of CotB was used. Both the N-terminal and the sandwich fusions were properly assembled in the coat structure \cite{32}. At least in the CotB-TTFC case, it was then possible to conclude that the site of insertion of the passenger protein in the carrier does not affect display on the spore surface. Western blot and FACS analysis were used to show that recombinant spores containing either one of the three types of fusions exposed TTFC at their surface, while \textit{in vivo} experiments showed that spore-displayed TTFC was able to induce a TTFC-specific immune response in a murine model and, therefore, was exposed in a biologically active form \cite{32}. Surface display of TTFC fused to CotB provided validation that the spore coat of \textit{B. subtilis} can be engineered to incorporate bioactive molecules of large molecular mass (TTFC is 51.8 kDa). TTFC-specific immune response induced by spores displaying the \textit{CotBA}_{105}-TTFC chimera (C-terminal fusion) was analyzed in more detail in subsequent studies. Mice orally or nasally immunized with recombinant spores induced fecal slgA and serum IgG at protective levels, and mice were protected when challenged with purified tetanus toxin \cite{41}. Similar spores were used in a mucosal-priming–parenteral-boosting vaccination strategy \cite{42}. Recombinant spores were used to orally prime BALB/c and C57BL/6 mice that were then subcutaneously boosted with soluble TTFC (without adjuvant). Protective levels of TTFC-specific IgA and IgG were observed also in this case \cite{42}. In a different study, recombinant spores displaying the \textit{CotBA}_{105}-TTFC chimera (C-terminal fusion) were shown to induce spleen and mesenteric lymph node cell proliferation as well as production of gamma interferon (IFNγ), but not of interleukin 4 (IL-4) and IL-10, in both locations, indicating that recombinant spores preferentially induce a strong cell-mediated immune response with a Th1 phenotype independently from their ability to germinate in the gastrointestinal tract \cite{43}. In more recent studies, CotB has been used as a carrier to display other antigens: the domains 1b-3 and 4 of the protective antigen (PA) of \textit{Bacillus anthracis} \cite{44}, the C-terminal part of the alpha toxin (CPA) of \textit{Clostridium perfringens} \cite{45}, the UreA protein of \textit{Helicobacter acinonychis} \cite{46}, the carboxy-terminal repeat domains of toxins A and B of \textit{Clostridium difficile} \cite{47}, and the VP28 protein of the white spot syndrome virus (WSSV) \cite{48} (Table 1). In all cases, recombinant spores were shown to induce antigen-specific and protective immune responses. While the display of \textit{H. acinonychis} and WSSV antigens was achieved by a C-terminal fusion to the truncated form of the carrier, \textit{CotBA}_{105} display of \textit{B. anthracis}, \textit{C. perfringens}, and \textit{C. difficile} antigens was obtained by fusing the heterologous molecules to the C-terminus of a full-length version of CotB. The truncated form of CotB has also been used to express a peptide formed by 18 histidine (18xHis) residues and recombinant spores used to adsorb nickel ions \cite{49}. Metal binding was not affected by either pH or temperature; the number of spores present was the only factor influencing nickel adsorption. In addition, approximately 40% of bound nickel could be recovered from the spore surface \cite{49}. The efficiency of metal binding, the robustness of the spore, and the possibility of recovering bound nickel suggest that recombinant spores are a potentially powerful bioremediation tool for the treatment of contaminated ecosystems \cite{49}.

\textbf{CotC as a Carrier Protein}

CotC is a 12-kDa protein in the \textit{B. subtilis} coat, initially identified by reverse genetics \cite{50} and then shown to be close to the surface based on genetic evidence \cite{51}. CotC was selected as a carrier candidate for its relative abundance in the coat. CotG, CotD, and CotC represent about 50% of the total solubilized coat proteins by mass. Such relatively high amounts could allow for the assembly of a significant number of CotC-based fusions on the coat, thus ensuring an efficient heterologous display. Expression of the \textit{cotC} gene is under the control of the mother cell-specific σ factor, σK, and the transcriptional regulators GerE and SpoIIIID. \textit{cotC} expression is also positively controlled by GerR in an indirect way, through the activation of SpoVIF, which stabilizes the transcriptional activator GerE and consequently
induces the expression of the GerE-dependent genes (34). The primary product of the cotC gene is a 66-amino-acid polypeptide extremely rich in tyrosine (30.3%) and lysine (28.8%) residues (50). However, it has been shown that CotC is assembled into at least five distinct protein forms, ranging in size from 12 to 30 kDa (36, 52). Two of these, having molecular masses of 12 and 21 kDa and corresponding most likely to a monomeric and homodimeric form of CotC, respectively, are assembled on the spore surface right after their synthesis 8 h after the onset of sporulation. The other two forms, 12.5 and 30 kDa, are probably the products of posttranslational modifications of the two early forms, occurring directly on the coat surface during spore maturation (36). The 23-kDa CotC species has been shown to be a CotC-CotU heterodimer (52), formed under the control of the spore coat protein CotE (53). As for CotB, CotC strictly requires cotH for its assembly, and none of the CotC forms is assembled in the coat of cotH spores. In a wild-type strain, CotC does not accumulate in the mother cell compartment, while in a mutant strain that overproduces CotH, at least two CotC forms, of 12 and 21 kDa, were found in the mother cell compartment. This supports the hypothesis that CotH, or a CotH-controlled protein, protects CotC from proteolysis prior to its assembly (37). In the case of CotC, only C-terminal fusions have been constructed with the two model antigens used with CotB, TTFC, and LTB (33). Both CotC-TTFC and CotC-LTB gene fusions were obtained by cloning tetC or eltB in frame with the last cotC codon under the transcriptional and translational control of the cotC promoter region. The gene fusion was then integrated into the B. subtilis chromosome at the amyE locus by double crossover recombination (33). Both of these fusion proteins were assembled on the coat of recombinant spores without major effect on the spore structure and/or function. A quantitative determination of recombinant proteins exposed on B. subtilis spores revealed that ca. 9.7 × 10^2 and 2.7 × 10^3 molecules of CotC-TTFC and CotC-LTB, respectively, were extracted from each spore. Although CotC appears more abundant than CotB within the coat, comparable amounts of heterologous proteins are exposed by the CotC- and CotBA_165-based systems. A possible explanation for this unexpected result comes from the finding that the C-terminal end of CotC is essential for the interaction of a CotC molecule with other CotC molecules and with other coat components (36). Support for this explanation also comes from the observation that when a heterologous peptide is fused at the N-terminal end of CotC, a normal pattern (four protein forms) of CotC assembly is observed and the efficiency of display is 5-fold increased (54). As in the case of CotB, CotC has been used as a carrier for a variety of heterologous proteins (Table 1). In addition to TTFC and LTB (33, 54), other antigens such as the domains 1b-3 and 4 of the PA of B. anthracis (44), the C-terminal repeats of toxins A and B of C. difficile (47), the VP28 protein of WSSV (48), and the UreA protein of H. acinonychis (46), described above as passengers fused to CotB, were also fused to the C terminus of CotC (Table 1). Human serum albumin (HSA) (55), the T-helper cell epitope Pep23 (KDSWTVNDIQKLVGK, amino acid residues 249 to 263) of the HIV1-RT (56), the tegumental protein TP22.3 of the human parasite Clonorchis sinensis (57), the envelope glycoprotein GP64 of the nucleopolyhedrovirus of the model lepidopteran Bombyx mori (BmNPV), and the alcohol dehydrogenase of B. mori (58, 59) have also been displayed on the spore surface by using CotC as a carrier (Table 1). With the use of UreA as a passenger, CotB and CotC were compared for their respective efficiency in anchoring the passenger to the spore surface (34). Although CotC was about 10-fold more efficient than CotB in expressing the passenger within the coat, it was not as efficient as CotB in displaying UreA on the spore surface, suggesting that CotB is an optimal carrier when external exposure is required, whereas CotC is better suited when a higher amount of passenger protein is needed (46).

** CotG as a Carrier Protein**

CotG is a 24-kDa protein produced in the mother cell compartment of the sporangium around hour 8 of sporulation under the control of the mother cell-specific σ factor, σ^K, and of transcriptional regulator GerE. Like cotC, cotG expression is indirectly controlled by GerR, through the activation of SpovIF, which positively acts on GerE and on GerE-dependent genes (34). CotG is assembled on the forespore surface as two main forms of about 32 and 36 kDa. The 32-kDa form most likely represents the unmodified product of the cotG gene (24 kDa) whose abnormal migration may be attributed to its unusual primary structure, characterized by the presence of 9 repeats of 13 amino acids (60) or, alternatively, as 7 tandem repeats of 7 and 6 amino acids, followed by 5 repeats of 7 amino acids (61). At the DNA level, this section of the cotG sequence consists of 19 likely paralogous segments of two different sizes: 12 21-bp-length copies and 7 18-bp-length copies (61). It has been proposed that the modular structure of cotG is the outcome of several rounds of gene elongation events of
an ancestral module (61). It is interesting to note that in all CotG-containing species, CotG preserves a modular structure, although the number and the length of the repeats differ (61). The 36-kDa form of CotG could be generated by extensive cross-linking of the protein as it is assembled into the spore coat. The view that CotG is able to form cross-links is suggested by analysis of the coat structure of sodA mutant spores (62). Spores produced by cotG mutants are not affected in their germination properties or resistance to lysozyme (60). As in the cases of CotB and CotC, CotG strictly requires cotH expression for its assembly (63). CotG controls the conversion of the CotB-46 form into the form of 66 kDa (CotB-66) found in mature spores (35). In the first attempt to use CotG as an anchoring motif, the biotin-binding protein streptavidin was fused to the C-terminal part of CotG (Table 1). The coding sequence for the 62-kDa, thus suggesting that the elimination of binding protein streptavidin was fused to the C-terminal expression for its assembly (64). In the case of CotB and CotC, CotG strictly requires cotH expression for its assembly (63). CotG controls the conversion of the CotB-46 form into the form of 66 kDa (CotB-66) found in mature spores (35). In the first attempt to use CotG as an anchoring motif, the biotin-binding protein streptavidin was fused to the C-terminal end of CotG (Table 1). The coding sequence for the streptavidin secretion signal was not included in the gene fusion, and a linker (Gly-Gly-Gly-Gly-Ser) was inserted between carrier and passenger (64). The gene fusion was expressed under the control of cotG promoter but, in contrast to the previously discussed cases of spore display, it was present on an extrachromosomal plasmid (62). Although the genetic stability of the gene fusion was not directly assessed, recombinant spores displayed active streptavidin on their surface, as demonstrated by FACS and fluorescence microscopy experiments (62). A similar plasmid-based approach was used to express a fusion of the C-terminal end of CotG to a variant form of the GFP protein of Aequorea victoria, GFPuv (65), a cofactor containing ω-transaminase of Vibrio fluvialis (66) and a N-acetyl-l-neuraminic acid (Neu5Ac) aldolase (67). In these cases, the same Gly(4)-Ser linker was inserted between CotG and the heterologous part. Neu5Ac aldolase was expressed using both a low- and a high-copy-number plasmid. An about 5-fold increase of the spore-associated enzymatic activity was obtained with the high-copy-number plasmid (67). The β-galactosidase of E. coli (68), the phytase of B. subtilis (69), and the UreA of H. acinonychis (46) were also fused to the C terminus of CotG, but those chimeras were encoded by recombinant DNA integrated into the B. subtilis chromosome. In the case of UreA a dot-blot analysis determined that about 5.3 × 10^3 UreA molecules were present in the spore coat (46). As summarized in Table 1, CotG has been mainly used as a carrier of passengers with enzymatic activity. Some of the CotG-displayed enzymes are either active as a dimer (ω-transaminase; 66) or a tetramer (streptavidin; 62), thus suggesting that the elimination of the cell wall translocation step for surface display is a clear advantage of spore-based over cell-based systems.

**OxdD as a Carrier Protein**

OxdD is a 43-kDa minor component of the spore coat with oxalate decarboxylase activity, therefore capable of converting oxalate into formate and CO₂ (70). The oxdD gene is transcribed during sporulation from a promoter recognized by σ^K and is negatively regulated by GerE (71). Therefore, OxdD is produced in the mother cell compartment of the sporangium, and its assembly within the coat occurs in a safA-dependent manner (72). Genetic and cell biological analyses suggest that OxdD is at an internal location within the coat, not at the surface. Because of its internal location, OxdD has been proposed as a carrier able to provide a high degree of protection to the passenger protein by burying the passenger protein below the surface (69). OxdD has been tested as a carrier by fusing to its C terminus a phytase (Phy) and a β-glucuronidase (β-Glu) (69). Phytases are monomeric enzymes widely used in animal nutrition. A B. subtilis phytase and an E. coli β-glucuronidase, encoded by the uidA gene, have been used as model enzymes for spore coat expression (69). In both cases a 10-amino-acid (10×Ala) linker was inserted between carrier and passenger (69). Recombinant spores carrying either the cotG::phy or the cotG::uidA gene fusions showed phytase or β-glucuronidase activity, indicating that OxdD is a suitable carrier for coat expression (69). In the case of spores displaying OxdD-Phy, a specific activity of 2.7 × 10^3 U/g (dry weight) was measured, indicating that 0.4 g of spores (dry weight) would represent 1,000 U of phytase, an amount of enzyme that, if added to 1 kg of feed in a daily swine diet, would replace 1 g of inorganic phosphorus supplementation (69). Spores expressing the phytase fused to OxdD or to CotG were then compared for their respective efficiency of surface expression. The phytase fused to OxdD was less efficiently displayed but more enzymatically active than the phytase fused to CotG, consistent with the possibility that, when fused to OxdD, the enzyme is covered by external coat layers (69). Protection of heterologous enzymes is an additional feature of the display system that could be relevant for some applications. For example, OxdD-Phy-expressing spores have been proposed as probiotics for animal use (69). Orally ingested spores of B. subtilis are known to perform an entire cycle of sporulation, germination, growth, and sporulation in the mammalian gastrointestinal tract, transiting unaffected through the highly acidic environment of the stomach, germinating in the upper part of the intestine, and resporulating in the lower intestinal region (73). In this context, the use of spores expressing OxdD-Phy in animal probiotic...
products could provide the further advantage of supply of usable phosphorus due to the continuous presence of active enzyme in the intestine (69).

SpsC as a Spore-Anchoring Signal

SpsC is a 389-amino-acid protein encoded by the third of the 11-gene sps operon. The operon is transcribed by a σ^K-controlled promoter and is positively regulated by the GerE transcriptional factor (74). Expression of the sps operon is involved in the biosynthesis of spore coat polysaccharides (74, 75). At its N terminus, at positions 6 to 10, SpsC contains the sequence Asn-His-Phe-Leu-Pro, proposed as a spore-anchoring motif (76). That sequence is preceded by two basic amino acids (Lys-Arg), a possible cleavage site for a trypsin-like protease. This observation has provided support for the hypothesis that during sporulation, proteolytic cleavage of a cytoplasmic form of SpsC is a necessary step (76). The sequence Asn-His-Phe-Leu-Pro was identified by screening phage display peptide libraries for peptides able to bind tightly to spores of B. subtilis (76). All the peptides isolated in this study contained the sequence Asn-His-Phe-Leu-Pro at the N terminus and exhibited clear preferences for Pro, at position 5. The peptide Asn-His-Phe-Leu-Pro was then shown to be sufficient for tight binding to spores of B. subtilis and of the closely related species B. amyloliquefaciens and B. globigii (76). Although this study was not specifically aimed at developing a surface display system, it highlighted that the SpsC protein or just the N-terminal peptide Asn-His-Phe-Leu-Pro can be used to display a passenger protein on the spore surface.

CotA as a Display System

CotA is a 65-kDa component of the outer layer of the B. subtilis coat (50, 51). CotA is responsible for the brown pigmentation that characterizes mature spores of B. subtilis when cultured on plates and which acts as a melanin-like pigment that protects the spore against UV radiation (77). CotA shows a high degree of sequence similarity with fungal and bacterial copper-dependent oxidases and was found to be a copper-dependent laccase that is active within the coat (77, 78). When assembled into the coat, CotA showed a half-life of inactivation at 80°C of about 4 h, indicating that it is a highly stable enzyme, naturally immobilized on the surface of the spore (78). The level of CotA at the spore surface could be increased with no detrimental effect on spore assembly or properties. Indeed, a strain bearing cotA on a multicopy plasmid formed colonies that were more pigmented than those of the wild type, and the spores had increased amounts of CotA and increased laccase activity (78). In a recent study (79), the laccase activity of CotA was used to perform directed evolution experiments on the spore coat. A plasmid-generated mutagenesis library of cotA was transformed into a B. subtilis mutant strain lacking the cotA gene, and spores of control and transformed strains were assayed for CotA activity with 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 4-hydroxy-3,5-dimethoxy-benzaldehydeazine (SGZ) as substrates. Spores of one mutant had a 120-fold increase in laccase activity (79).

Display on Spores of Bacilli Other Than B. subtilis

In addition to B. subtilis, other Bacillus species have been proposed as systems for the surface display of heterologous proteins. The 130-kDa Cry1Ac protoxin of B. thuringiensis is a major component of the spore coat, and its N terminus is found to be spore surface exposed (80). Based on this, Cry1Ac has been suggested as a suitable carrier for surface display on spores of B. thuringiensis (81). The system was initially developed by using the green fluorescent protein (GFP) as a model passenger. This identified the minimal region of the protoxin required for surface display. DNA coding for the carrier region of the protoxin was carried on a plasmid and, when fused to GFP, used to transform a Cry^− mutant strain, thus avoiding competition with the native protoxin (81). The system was used to successfully display a single-chain antibody (scFv) recognizing the hapten 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx) (81). Fluorescence microscopy experiments showed that the anti-phOx antibody was expressed and displayed on the B. thuringiensis spore surface (81). InhA, an exosporium protein of B. thuringiensis, was shown to be able to display proteins active either as a monomer (GFP) or as a tetramer (β-Gal) (82). This study then proposed that the exosporium, the surface structure surrounding the spore of some Bacillus species, would be a suitable surface for the display of heterologous proteins. Two components of the exosporium of B. anthracis, BclA and BclB, have also been proposed as carriers for spore surface display (83). BclA and BclB first assemble at the spore surface and then are proteolytically cleaved. Finally, the mature proteins become stably attached to the spore surface via their N termini (83). The initial N-terminal 19 residues (amino acids 2 to 24) of BclA, including the proteolytic cleavage site, targeted GFP to the spore surface. However, because released spores did not show fluorescence, the 19-amino-acid motif alone was not sufficient for attachment of the fusion protein to the mature exosporium (83).
SURFACE DISPLAY ON NONRECOMBINANT SPORES

All display systems based on the use of bacterial cells or bacteriophages, as well as the spore-based systems summarized in the previous sections, rely on the genetic engineering of the host. This is a major drawback when the application of the display system involves the release into nature of the recombinant host (field applications) and, in particular, when the display system is designed for human or animal use (delivery of antigens or enzymes to mucosal surfaces). Serious concerns over the use of live genetically modified microorganisms, their release into nature, and their clearance from the host following oral delivery have been raised (84). To overcome this obstacle, nonrecombinant approaches are highly desirable and their development is strongly encouraged by control agencies (84). In this context, various nonrecombinant approaches to display heterologous proteins on the spore surface have been recently proposed. In the first study suggesting that heterologous proteins can be adsorbed on the spore surface, the gene encoding the NADPH-cytochrome P450 reductase (CPR), a diflavin-containing enzyme, was overexpressed in B. subtilis cells by using the isopropyl-β-D-thiogalactopyranoside-inducible vegetative promoters PgroE and Ptac (85). The expressed CPR was released into the culture medium after sporulation by autolysis of the mother cell and was found associated to the spore surfaces. Purified spores showed CPR activity, and the enzyme was accessible to anti-CPR antibodies in FACS experiments (85). It is noteworthy that the enzyme used in this study contains two flavin cofactors (FMN and FAD) and, for this reason, cannot be produced or secreted by standard expression systems (85). Although this first study did not address the mechanisms involved in the spontaneous adhesion of the enzyme to the spore surface and did not investigate whether other enzymes had a similar behavior, it clearly suggested a new approach for spore display. A similar result was obtained using spores of B. thuringiensis. The endo-β-N-acetylglucosaminidase (Mbg) of B. thuringiensis is a putative peptidoglycan hydrolase containing two LysM domains at its N terminus and had been used to display GFP on the surface of B. thuringiensis cells (86). Fusions of Mbg to GFP and a bacterial laccase (WlacD) were found to associate with the spore surface, suggesting that Mbg is also a possible carrier for spore surface display (87).

Nonrecombinant Display of Heterologous Antigens and Enzymes

A different approach for spore adsorption was followed by Huang et al. (88). Previously purified proteins were mixed with purified spores of B. subtilis and adsorption conditions were developed. A collection of antigens (the TTFC of C. tetani, PA of B. anthracis, Cpa of C. perfringens described above, and glutathione S-transferase (Sj26GST) from Schistosoma japonicum (89)) were expressed in E. coli and adsorbed to spores. Adsorbed spores were able to induce specific and protective immune responses in mice immunized mucosally (88). Spore adsorption was more efficient when the pH of the binding buffer was acidic (pH 4) and less efficient or totally inhibited at pH values of 7 or 10 (88). A combination of electrostatic and hydrophobic interactions between spores and antigen were suggested to drive adsorption. Interestingly, adsorption was not dependent on any specific coat proteins but, rather, was due to the negatively charged and hydrophobic spore surface (88). In addition, the same study showed that killed or inactivated spores were equally effective as live spores in adsorbing the various antigens (88). A similar approach was also used to adsorb the phytase of E. coli to spores of a probiotic strain of B. polyfermenticus (90) and the β-galactosidase of Alicyclobacillus acidocaldarius to B. subtilis spores (91). In the case of the phytase, immobilization on the spore surface stabilized the enzyme by increasing its half-life at temperatures ranging between 60 and 90°C, but also caused a loss of activity of about 30% (91). The β-galactosidase of A. acidocaldarius was more stable when adsorbed to spores than the free enzyme at high temperatures and at low pH values (91). This study also reported that spores of mutant strains having an altered (or totally lacking) protein in the outer layer of the coat adsorbed enzymes much more efficiently than wild-type spores (91). This observation is consistent with results obtained with B. subtilis and B. anthracis spores (92) and suggests a negative effect of the spore outermost structures on adsorption to surface or molecules.

Spore Surface Properties and Adsorption

The physicochemical properties of the spore surface have been addressed in different studies with different approaches. An early study showed that spores of B. subtilis are negatively charged by time-resolved micropotentiometry (93). In an aqueous environment the spore behaves like an almost infinite ionic reservoir and can accumulate billions of protons (approximately 2 × 10¹⁰ per spore) (93). The carboxyl groups were identified as the major ionizable groups in the spore and, on the basis of the diffusion time analysis, it was found that proton diffusion is much lower in the spore core than within the coat and cortex (93). This, then,
suggested that the inner membrane, separating core from cortex and coat in a dormant spore, is probably a major permeability barrier for protons (93). The role of electrostatic forces in spore adhesion to a planar surface has been also addressed by studying spores of B. thuringiensis (94). The surface potentials of spore and mica surfaces were experimentally obtained using a combined atomic force microscopy (AFM)-scanning surface potential microscopy technique (94). By these techniques, the surface charge density of the spores was estimated at 0.03 μC/cm² at 20% relative humidity and decreased with increasing humidity. This work showed that the electrostatic force can be an important component in the adhesion between the spore and a planar surface (94). However, the interaction between spores and a protein in an aqueous environment may involve additional forces, as suggested by the observation that the enzyme β-galactosidase of A. acidocaldaricus binds more efficiently to modestly negatively charged mutant spores than to highly negatively charged wild-type spores (91). A recent study showed that the electric charge of wild-type and mutant spores can be measured at the single-spore level by using optical tweezers, allowing for a detailed analysis of the physical properties of the spore surface (95). Adhesion of B. subtilis and B. anthracis spores to abiotic surfaces has also been shown to be largely due to negative charges on the surfaces of spores of both species (which is notable given that, unlike B. subtilis spores, B. anthracis spores have an exosporium surrounding the coat). In both species, the spore surface thermodynamic properties changed in similar ways when the spore surface was altered due to mutations, and in general, adhesion increased in the mutants (96).

ADVANTAGES OF THE SPORE SURFACE DISPLAY SYSTEMS

Spore-based surface display systems provide several advantages with respect to other display approaches. A first advantage comes from the well-documented robustness of the Bacillus spore, which grants high stability to the display system even after a prolonged storage. This aspect has been tested with spores displaying CotBΔ105-TTFC. Aliquots of purified recombinant spores were stored at −80°C, −20°C, +4°C, and at room temperatures and assayed for the amount of heterologous protein present on the spore after different storage times. A dot-blot analysis with anti-TTFC antibody showed identical amounts of displayed TTFC at all time points (27). The stability of the display system is an extremely useful property for a variety of biotechnological applications. Heat stability, for example, is a stringent requirement in developing new mucosal vaccine delivery systems, especially in developing countries, where poor distribution and storage conditions are major limitations. High stability is also extremely useful in industrial and environmental applications, where (nonspore) cell-based systems are limited because most cells have poor long-term survival, especially under extreme conditions (97). The safety record of several Bacillus species (98) is another important advantage of spores over other systems. Several Bacillus species, including B. subtilis, are widely used as probiotics and have been on the market for human or animal use for decades in many countries (98). Although most studies in humans or animals have been performed with laboratory strains of B. subtilis, in some cases, intestinal isolates and strains with probiotic properties have also been used to display heterologous proteins (99). The safety of the live host is obviously an essential requirement if the display system is intended for human or animal use, such as delivery of vaccines or therapeutic molecules to mucosal surfaces. A limitation of cell- and phage-based display systems is the size of the heterologous protein to be exposed, since it may affect the structure of a cell membrane-anchoring protein or of a viral capsid. In addition, cell-based systems require a membrane translocation step in order to externally expose a protein produced in their cytoplasm (97). With the spore-based system, heterologous proteins are produced in the mother cell and their assembly on the spore surface does not involve membrane translocation (Fig. 4). Therefore, this system has fewer size limitations and, as described above, large proteins (for example, TTFC of 51.8 kDa) have been successfully displayed on the spore surface.

Spore surface display by the nonrecombinant approach has an additional advantage. A recent publication showed that the B subunit of the heat-labile toxin of E. coli (LTB) is exposed on the spore surface in its native pentameric form (100). This is an important observation because in other display systems LTB could only be displayed as a monomer. Because only the pentameric form of LTB binds its receptor, the ganglioside GM1, spore surface display allows enhanced activation of the immune system using this molecule (100).

FUTURE DEVELOPMENTS

A better understanding of coat protein assembly mechanisms should permit the design of improved strategies for the display of fusion proteins at the spore surface. An example is CotB: although it has been used to display
several passenger proteins, the presence of a wild-type copy of the *cotB* gene is needed for display to be efficient (32). Assembly of CotB involves a complex pattern of protein-protein interactions (35), leading to the hypothesis that a wild-type copy of CotB is able to recruit the CotB-based chimera to the coat. The identification of the CotB regions that undergo covalent modifications during assembly would be an important step in designing novel fusions that, as required for each application, would or would not be cross-linked to the spore. Some coat components have an enzymatic activity. In addition to CotA and OxdD discussed above, Tgl, a 24-kDa coat component, has been shown to have a transglutaminase activity (101, 102). In a mutant strain lacking *tgl*, some coat components are more easily extractable, suggesting that Tgl is involved in the cross-linking (and therefore in the insolubilization) of some coat proteins (101–103). Only some Tgl substrates have been identified (101, 102), and, also in those cases, the amino acid residues involved in the cross-linking reaction are not known. A better understanding of this process may be important for surface display. A passenger containing the appropriate signal could be covalently bound to a coat component (and therefore to the coat) in a Tgl-dependent way, allowing its controlled display. It has been recently discovered that the *B. subtilis* spore has an additional structure surrounding the outer coat layer. This outermost layer has been named the crust, and has been shown to be formed by proteins, probably in a glycosylated state (104, 105). The *cgeA* gene and genes of the *cotVWXWZ* cluster have been shown to be involved in crust formation, and CotZ and CgeA have been identified as essential crust components (105). Identification of the crust has been extremely relevant for the development of future spore display systems. Crust components, being on the surface of the spore, are obvious carrier candidates. Recently, CotZ has been fused to UreA of *H. acinonyx* (106) and to FlID of *C. difficile* (107), resulting in fusions that were efficiently exposed on the spore surface. Vectors to target heterologous proteins to the spore surface by using CgeA as a carrier have also been developed and shown to successfully display a *Helicobacter pylori* antigen (108).

Future developments of spore display systems will include the approach involving protein adsorption on nonrecombinant spores. Any future development of this technique must necessarily be based on a better understanding of the mechanisms involved in spore adsorption. A combination of electrostatic and hydrophobic interactions between spores and passengers has been suggested as being responsible for adhesion (88, 90). However, at least in the case of β-galactosidase adhesion to *B. subtilis* spores, the electrostatic force does not seem to play a predominant role (91). Whether other factors...
(e.g., van der Waals and capillary forces) are also involved, and how the involved forces are affected by external factors such as humidity or by properties of the passenger protein, are all relevant questions that still need to be addressed.

A future and exciting extension of recombinant and nonrecombinant spore display systems comes from the recent observation that spores of *B. subtilis* can be used as building blocks for stimuli-responsive new biomaterials (109). Spores displaying heterologous molecules can then be used to self-assemble submicrometer-thick monolayers and build functionalized surfaces able to catalyze a chemical reaction or sense a specific molecule.

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