The spore surface of intestinal isolates of *Bacillus subtilis*

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**Abstract**

*Bacillus subtilis* has been used for over 50 years as a model organism for biochemistry, genetic, molecular biology and cell biology studies. More recently, its spore has been proposed as a platform to display heterologous proteins and as a vehicle for mucosal vaccination. We characterize here the spore surface of four human intestinal strains of *B. subtilis*, previously identified as able to grow anaerobically and form biofilm. These properties, lost in laboratory strains, are relevant for the colonization of human mucosal sites and likely to improve the efficiency of strains to be used for mucosal delivery. Our characterization is an essential preliminary step for the development of these intestinal strains as display systems and has indicated that spores of at least one of them are more efficient than the laboratory strain for the non-recombinant display of two model heterologous proteins.

**Introduction**

Members of the *Bacillus subtilis* species are low-GC Gram-positive bacteria, not genetically monomorphic but divided into two distinct phylogenetic subgroups: the *B. subtilis* ssp. *subtilis* and *B. subtilis* ssp. *spizizenii* (Nakamura et al., 1999). The subspecies *subtilis* contains *B. subtilis* 168, a widely studied strain, considered the model system for spore formers. Both subspecies share the ability to differentiate an endospore (spore) when cell growth is no longer allowed because of nutrient starvation or other unfavourable environmental conditions. In that case, the vegetative cell undergoes an asymmetric cell division and produces a sporangium formed by a large mother cell and a small forespore (Stragier & Losick, 1996). The mother cell contributes to forespore maturation and undergoes autolysis at the end of the process, allowing the release of the mature spore into the environment (Stragier & Losick, 1996). In the presence of favourable environmental conditions the mature spore can germinate, generating a vegetative cell able to grow and, eventually, to re-sporulate. The processes of sporulation and germination have been reviewed recently (Dworkin & Shah, 2010; Higgins & Dworkin, 2012).

The spore is highly stable and resistant to environmental insults. Such properties are due to the peculiar structure of the spore, characterized by a dehydrated cytoplasm surrounded by a peptidoglycan-like cortex, a coat and a crust (McKenney et al., 2013). The coat is formed by more than 70 different proteins organized into three layers, and the recently identified crust is made of proteins and glycans (McKenney et al., 2013). For its stability and structural properties, the spore of *B. subtilis* has been proposed as a platform to display heterologous molecules (Istitico et al., 2007; Cutting et al., 2009; Knecht et al., 2011). A variety of antigens and enzymes have been displayed on the spore surface by either a recombinant or a non-recombinant approach (Istitico & Ricca, 2014). In addition to various antigens and enzymes have been displayed on the spore surface by either a recombinant or a non-recombinant approach (Istitico & Ricca, 2014). In addition to various antigens and enzymes (Huang et al., 2010; Istitico et al., 2013), the non-recombinant approach has been used to display an enzyme (Sirec et al., 2012) and virions (Song et al., 2012). Non-recombinant adsorption to the spore surface has been attributed to a combination of electrostatic and hydrophobic forces (Huang et al., 2010), but at least for the adhesion of the beta-galactosidase of *Alicyclobacillus acidocaldarius*, the electrostatic forces have not been found as major determinants of adsorption (Sirec et al., 2012). For the safety record of several species of spore formers, including *B. subtilis*, and for the ability to induce protective and antigen-specific immune responses, spores are now considered a powerful mucosal vaccine vehicle.
Spore coat of intestinal *B. subtilis*

(Ciabattini *et al.*, 2004; Cutting *et al.*, 2009). Most of the immunization studies on animal models have been performed using PY79, an extremely well characterized laboratory collection (domesticated) strain of *B. subtilis* sp. *subtilis* derived from strain 168 (Youngman *et al.*, 1984).

Although aerobic spore-formers have been generally considered to be soil bacteria (Fritze, 2004), recent reports have clarified that these organisms are normal inhabitants of the human and animal gut and that, in a murine model, ingested spores undergo an entire life cycle, germinating in the upper part of the intestine, duplicating and sporulating again in the lower intestinal tract (Cutting *et al.*, 2009). We have preliminary characterized several *Bacillus* strains isolated from ileal biopsies of healthy volunteers and found that, unlike domesticated strains, some of them produce a biofilm and grow in anaerobic conditions (Fakhry *et al.*, 2008). As these properties are relevant for the adhesion and colonization of mucosal sites it is reasonable to predict that spores of some wild strains could perform better than domesticated strains as vaccine vehicles, either directly delivering antigens or expressing antigens on the cell surface after spore germination in the gut.

Here we report the analysis of the spore surface of four of these intestinal isolates belonging to *B. subtilis* species (Fakhry *et al.*, 2008). Such an analysis is an essential first step for the use of these strains for mucosal vaccine delivery. Indeed, the recombinant display approach is based on the use of spore surface proteins to anchor antigens and the non-recombinant approach takes advantage of the electric charge and hydrophobic properties of the spore surface. Our results indicate that the analyzed isolates can be used to anchor heterologous antigens and that at least one of them is more efficient than the domesticated strain in adsorbing two model proteins.

**Material and methods**

**Bacterial strains, sporulation and spore purification**

*Bacillus subtilis* strains used in this study were the laboratory strain PY79 (Youngman *et al.*, 1984) and four intestinal isolates, SFB2, SF106, SF128 and SF195 (Fakhry *et al.*, 2008). Sporulation was induced in Difco sporulation (DS) medium by the exhaustion method (Cutting & Vander Horn, 1990). After a 30-h incubation at 37 °C, spores were collected, washed four times, and purified by water-washing using overnight incubation in H2O at 4 °C to lyse residual sporangial cells (Nicholson & Setlow, 1990). Spore purity was checked by microscope inspection and was higher than 95%.

**DNA extraction, PCR and sequencing of the gyrA gene**

Exponentially growing cells were used to extract chromosomal DNA as previously reported (Cutting & Vander Horn, 1990). gyrA fragments from nucleotide positions 43–1065 (Chun & Bae, 2000) were PCR-amplified by using chromosomal DNA as a template and oligonucleotides gyrA-F (5'-CAG TCA GGA AAT GCG TAC GTC CTT-3') and gyrA-R (5'-CAA GGT ATT GCT CCA GCC ATT GCT-3') to prime the reaction. Amplified DNA fragments were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions (BMR Genomics, Padua, Italy). gyrA gene sequences have been deposited in GenBank-NCBI database with the accession numbers KJ921799–KJ921802. Multiple sequences were aligned using the program CLUSTAL X, version 1.8. Phylogenetic analysis and genetic trees were performed using PHYLOGENY.FR platform (Dereeper *et al.*, 2008).

**Hydrophobicity assays**

The BATH assay (Wiencek *et al.*, 1990) was used to assess the relative hydrophobicity of bacterial spores. Briefly, spore suspensions (1.5 ± 0.1 x 10⁸ counted under the optical microscope with the Bürker chamber) were incubated for 15 min at 25 °C. Then, 1.0 mL of hexadecane (Sigma-Aldrich) was added to 3.0 mL of each spore suspension. The mixture was vortexed for 1 min in glass test tubes (15 x 100 mm), and the hexadecane and aqueous phase allowed to partition for 15 min. The aqueous phase was carefully removed with a Pasteur pipette and the OD measured at 440 nm. As previously reported (Wiencek *et al.*, 1990), the decrease in OD₄₄₀ of the aqueous suspension indicated the relative hydrophobicity and was calculated as follows: 100 (A₀ – A₁)/A₀, where A₀ and A₁ were the initial and final OD₄₄₀, respectively.

**Purification of beta-galactosidase (**β**-Gal) and LTB**

Expression vectors containing the structural genes for β-Gal of *Alicyclobacillus acidocaldarius* and LTB of *Escherichia coli* have been described previously (Sirec *et al.*, 2012; Istitaco *et al.*, 2013b). Expression of these two genes was induced by 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) in *E. coli* BL21 (DE3) cells (Novagen) and over-produced β-Gal and LTB purified using the GST-tag or His-tag, respectively, as described by the manufacturer (GE Healthcare Life Science). The two proteins were purified under native conditions using specific column as recommended by the manufacturer (GE Healthcare Life Science)
Adsorption reaction and enzyme detection

For LTB adsorption assay, 2 μg of purified antigen was added to a suspension of 2 × 10⁹ spores in 0.15 M phosphate-buffered saline (PBS) pH 4.0 at 25 °C in a final volume of 200 μL. After 1 h of incubation, the binding mixture was centrifuged (10 min at 13 000 g) to fractionate pellet and supernatant for further experiments. For β-Gal adsorption reaction, the enzyme was added to a suspension of 2 × 10⁹ spores in 50 mM sodium citrate, pH 4.0 at 25 °C in a final volume of 200 μL. After 1 h of incubation, an aliquot (70 μL) of the binding mixture was stored at 4 °C and the remaining part of the binding mixture was centrifuged (10 min at 13 000 g) to fractionate pellet and supernatant. All fractions were then used for β-Gal assays: 20 μL of each fraction were added to the reaction buffer (50 mM sodium citrate buffer at pH 5.5, 2NP-β-d-Gal 10 mM) and mixtures incubated at 65 °C for 5 min; the reaction was then blocked by addition of 800 μL of 1 M Na₂CO₃. When the assay was performed on samples containing spores, the samples were centrifuged prior to measurement of optical density at 420 nm. We expressed results of enzymatic assays in total units, where 1 unit is defined as an amount of β-Gal able to hydrolyze 1 μmol of substrate in 1 min at standard conditions (Sirec et al., 2012). The final data are the average of results of three independent experiments.

Western and dot-blot analysis

Extraction of proteins from mature spores was performed with treatment at 65 °C in SDS-dithiothreitol (DTT) extraction buffer or at 4 °C in 0.1 M NaOH (Isticato et al., 2010). The insoluble fraction of coat proteins was extracted by a decoating procedure (Vepachedu & Setlow, 2005). Western blot analyses were performed by standard procedures. Nitrocellulose membranes were used for electrophoresis and proteins and then blotted with anti-CotZ, anti-CotB, anti-CotG or anti-CotC antibodies as described previously (Isticato et al., 2013a). A quantitative determination of the amount of LTB was obtained by dot blot experiments with specific anti-LTB antibodies analyzing serial dilutions of purified LTB and the supernatant of binding assays (Isticato et al., 2013b). Filters were then visualized by the ECL-prime (Amersham Pharmacia Biotech) method and subjected to densitometric analysis by Fluor-S Multimager (Bio-Rad) (Isticato et al., 2013b).

Immunofluorescence microscopy

Aliquots of 2.0 × 10⁶ LTB-adsorbed spores were pretreated with 1% bovine serum albumin (BSA) – 1 x PBS, pH 4.0 for 30 min prior to incubation overnight a 4 °C with the polyclonal anti-LTB antibodies (rabbit) diluted 1 : 70 in PBS–1% BSA. As a control of the specificity of this technique, spores alone were reacted with anti-LTB antibodies. After three washes, the samples were incubated with a 64-fold diluted anti-rabbit secondary antibody conjugates with Tetramethyl Rhodamine, TRITC (Santa Cruz Biotechnology, Inc.) and washed four times with PBS. Washed samples were resuspended in 20 μL of PBS and 10 μL analyzed using immunofluorescence microscopy as described previously (Manzo et al., 2013).

Results and discussion

Phylogenetic position of the intestinal isolates of B. subtilis

Strains SFB2, SF106, SF128 and SF195 have been identified previously as members of the B. subtilis species by biochemical tests and analysis of the 16S rDNA gene (Fakhry et al., 2008). These approaches did not allow the assignment of the four strains to one of the two subspecies of the B. subtilis group (Nakamura et al., 1999). We therefore decided to analyze the nucleotide sequence of the gyrA gene, which has been used to discriminate between the two subspecies (Chun & Bae, 2000; Earl et al., 2008). A 1022-base pairs fragment of the gyrA gene was PCR amplified, sequenced and the obtained data used to construct a phylogenetic tree. As shown in Fig. 1, all strains belong to the B. subtilis ssp. subtilis, with SF128 slightly separated by the other three intestinal isolates.

Protein composition of the spore surface

To analyze the protein composition of the spore surface of the four isolates, we used purified spores to extract and characterize the pattern of coat proteins released after SDS-DTT or NaOH treatment. These two procedures are standard methods to extract the majority or only the alkali-soluble fraction of spore surface proteins, respectively (Nicholson & Setlow, 1990). As reported in Fig. 2a, all strains showed a similar pattern of extracted proteins. Minor differences were observed in the relative abundance of some proteins, such as the 70- and 32-kDa proteins, most likely corresponding to CotA, and to one of the CotG forms, respectively (Fig. 2a). After the treatment with NaOH, the pattern of released coat proteins presented more differences between PY79 and intestinal
isolates with proteins present in some strains and totally lacking in others (arrowhead in Fig. 2b).

To evaluate the possibility of using the intestinal strains as vaccine vehicles we used a Western blot approach to verify whether proteins most commonly used as carriers to anchor heterologous antigens on the spore surface were present in the wild strains. We then analyzed by Western blot the surface proteins using antibodies raised against the coat proteins CotB, CotC and CotG or against the crust protein CotZ, all previously used to display antigens (Isticato & Ricca, 2014). As reported in Fig. 3, CotB, CotG and CotZ were present in all strains analyzed. In all cases, CotB was mostly present in its mature form of 66 kDa (Zilhao et al., 2004). In laboratory strain PY79, the conversion of the CotB immature form of 46 kDa into the mature form is known to depend on the presence of both CotH and CotG. The presence of only the 66-kDa form of CotB in the four intestinal isolates is presumably indicative also of the presence of active CotH and CotG. The presence of the CotG in these strains was confirmed also by Western blot with anti-CotG antibody. CotZ is a crust component and its presence on the spore surface is considered indicative of crust formation (McKenney et al., 2013). As shown in Fig. 3, CotZ is present in all strains, suggesting that all intestinal strains form the crust.

As previously reported, antibodies raised against CotC specifically recognized six CotC-dependent polypeptides in Western blot analyses of proteins extracted from PY79 (Isticato et al., 2004). One of these six bands, the 17-kDa one, corresponds to the CotC-homologue CotU, also recognized by anti-CotC antibody (Isticato et al., 2008). The 21- and 23-kDa proteins are, respectively, a CotC-CotC homodimer and a CotC-CotU heterodimer, the latter formed only when CotE is in direct contact with CotC and CotU (Isticato et al., 2010). The 12.5- and the 30-kDa proteins are probably the result of post-translational modifications of CotC that occur only on the spore coat (Isticato et al., 2008). Only for strain SF106 did the pattern of CotC-CotU proteins appear similar to that of PY79, whereas that for the other three strains differed: (1) SFB2 has a single protein corresponding in size to the CotC-CotU heterodimer of 23 kDa; (2) SF128 has a pattern similar to that of PY79 but the 12.5-, 17- and 30-kDa proteins migrate faster than in PY79, suggesting differences in the size and/or in the post-translational modifications of the proteins; (3) in SF195 the antibody recognized only three bands, probably corresponding to CotC and CotU monomers and to CotC heterodimer. Those variations in the pattern of CotC-CotU proteins are intriguing and their detailed analysis is likely to provide insights on the mechanism of homo- and heterodimer
formation. However, these interesting aspects are outside the scopes of the present study that is aimed at evaluating the possibility of using the selected four wild strains for antigen display. Within this frame, the experiments of Fig. 3 allow us to conclude that at least three of the spore surface proteins most commonly used as carriers – CotB, CotG and CotZ (Isticato & Ricca, 2014) – are present in all four intestinal strains and are therefore potentially usable for heterologous display on the spore surface of these strains.

**Spore surface properties and non-recombinant display**

The efficiency of non-recombinant adsorption on the spore surface has been associated to the physico-chemical properties of the spore and in particular to its relative hydrophobicity (Huang et al., 2010; Sirec et al., 2012). We used the BATH assay (Wiencek et al., 1990) to compare the hydrophobicity of spores of PY79 and of the intestinal strains. Spores were suspended in water, vigorously mixed with hexadecane, and the two phases allowed to separate. Hydrophobic spores accumulate at the interface between water and the solvent and, as a consequence, the number of spores remaining in the aqueous phase decreases. This decrease is an indication of the hydrophobicity of the spore and its inverse is given as a percentage of relative hydrophobicity (Wiencek et al., 1990). As shown in Fig. 4a, spores of PY79 and of the intestinal isolates showed minimal and not statistically significant differences in their percentage of hydrophobicity.

We then analyzed the ability of spores of the intestinal isolates to adsorb two model heterologous proteins for which we have previously determined their adsorption characteristics to spores of strain PY79: the β-Gal of *Alicyclobacillus acidocaldaricus* (Sirec et al., 2012) and the B subunit of the heat-labile toxin (LTB) of *E. coli* (Isticato et al., 2013b). As previously described (Sirec et al., 2012), purified β-Gal (0.5 µg) was incubated with PY79 spores and the adsorption mixture either assayed for β-Gal activity (black bars in Fig. 4b) or fractionated by centrifugation. The pellet and supernatant fractions were then assayed independently (Fig. 4). In agreement with previously reported data (Sirec et al., 2012), about 50% of the β-Gal activity was found in the supernatant when PY79 spores were used (unbound enzyme, white bars in Fig. 4b).
other 50% of the β-Gal activity was found in the pellet fraction (spore-bound enzyme, grey bars in Fig. 4b). Interestingly, more than 80% of β-Gal activity was found associated with the pellet fraction in the case of strains SFB2 and SF106 (Fig. 4b). These results strongly indicate that these wild strains adsorbed β-Gal more efficiently than PY79 spores did. With spores of strains SF128 and SF195 the total β-Gal-specific activity was dramatically reduced, suggesting that the enzyme was degraded or inactivated in the presence of those spores (Fig. 4b).

As previously described (Isticato et al., 2013b), purified LTB (2.0 μg) was incubated with spores (2.0 × 10⁸) and the adsorption reaction fractionated by centrifugation. The supernatant fraction with unbound, free LTB was analyzed by dot blot with anti-LTB antibody, and the pellet fraction, containing spores and LTB bound to them, was used to extract and analyze proteins by Western blot with anti-LTB antibody. A densitometric analysis of the dot blot of Fig. 5a indicated that, similarly to what has been reported previously (Isticato et al., 2013b), the amounts of LTB left unbound by PY79 spores was approximately 6% (128 ± 12.5 ng) of the total amount of LTB. A similar value was obtained for SFB2 spores (4%, 81 ± 15.5 ng). SF128 and SF195 spores behaved differently and were less efficient than PY79 spores, leaving unbound approximately 40% (967 ± 23.3 ng) and 13% (255 ± 17.8 ng) of LTB, respectively. Conversely, SF106 spores did not leave any LTB in the supernatant, suggesting that spores bound all the LTB present or that LTB was degraded (Fig. 5a). Figure 5b reports the semi-quantitative analysis by Western blot of the pellet fractions and indicates that LTB could be extracted from PY79, SFB2, SF106 and SF195 but not from SF128 spores. Results shown in Fig. 5b indicate that SF128 spores either tightly adsorb or degrade LTB and that SF106 spores do not degrade all the LTB present. We used an immunofluorescence approach with anti-LTB antibody to visualize LTB on the spore surface and to compare the amount of LTB present on PY79 and SF106 spores. The fluorescence signal is more intense around SF106 than around PY79 spores, suggesting that LTB is not degraded by SF106 spores but is, instead, more efficiently adsorbed on SF106 than PY79 spores (Fig. 6). The results shown in Figs 4–6 suggest that: (1) in spite of a similar hydrophobicity,
SF106 spores adsorb both β-Gal and LTB more efficiently than the domesticated strain PY79; and (2) only part of LTB adsorbed on SF106 spores can be extracted.

Conclusions

The main result of this work is the finding that intestinal isolates of B. subtilis can be used for recombinant and non-recombinant spore surface display of heterologous proteins. As these intestinal isolates can grow anaerobically and form biofilm, they are likely to colonize the human intestinal mucosa better and, as a consequence, to perform better as mucosal vaccine vehicles, compared with domesticated strains. CotB, CotG and CotZ, three spore surface proteins commonly used for the recombinant anchoring of heterologous proteins on the spore surface, are present in the wild strains and can be potentially used as carriers for spore surface display. Fusions based on the use of such carriers could then be moved into the intestinal isolates by chromosomal DNA-mediated transformation or by transduction (Singh et al., 2013). Two model heterologous proteins, an enzyme and an antigen, have been displayed by a non-recombinant approach on spores of the intestinal strains. One of the intestinal strains, SF106, turned out to be more efficient than the laboratory strain in displaying both heterologous model proteins.

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References


