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Display of the peroxiredoxin Bcp1 of *Sulfolobus solfataricus* on probiotic spores of *Bacillus megaterium*

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ABSTRACT

Bacterial spores displaying heterologous proteins have been proposed as a safe and efficient method for delivery of antigens and enzymes to animal mucosal surfaces. Initial studies have been performed using *Bacillus subtilis* spores, but other spore forming organisms have also been considered. *B. megaterium* spores have been shown capable of displaying large amounts of a model heterologous protein (*Discosoma* red fluorescent protein mRFP) that in part crossed the exosporium to localize in the space between the outer coat layer and the exosporium. Here, *B. megaterium* spores have been used to adsorb Bcp1 (bacterioferritin comigratory protein 1), a peroxiredoxin of the archaeon *Sulfolobus solfataricus,* known to have an antioxidant activity. The spores were highly efficient in adsorbing the heterologous enzyme which, once adsorbed, retained its activity. The adsorbed Bcp1 localized beneath the exosporium, filling the space between the outer coat and the exosporium. This unusual localization contributed to the stability of the enzyme-spore interaction and to the protection of the adsorbed enzyme in simulated intestinal or gastric conditions.

Introduction

The delivery of drugs and antigens by the oral or nasal routes offers several advantages over parenteral administration and is gaining increasing relevance for the treatment of human and animal diseases. Mucosal routes are promising alternatives to delivery by injection, because the high vascularization of the mucosal surfaces allows the direct transfer of molecules into the systemic circulation [1]. However, the number of drugs and antigens that can be effectively administered by the oral or nasal route is severely limited by the rapid loss of activity encountered by many of these molecules at mucosal sites. Therefore, the successful development of mucosal therapeutic molecules relies on efficient delivery systems, able to stabilize and protect the molecules from degradation and to reduce or avoid completely the loss of biological activity [2]. A variety of drug delivery systems has been proposed, including live microorganisms, virus particles, synthetic nanoparticles, liposomes, microspheres, gels and cyclodextrins [2,3]. Bacterial spores displaying heterologous proteins have also been proposed as a tool for the delivery of molecules to mucosal surfaces [4,5]. Spores are extremely stable and are potentially able to combine some advantages of live microrganisms with those of synthetic nanoparticles [4,5].

Bacterial spores are mainly formed by Gram-positive organisms of different genera and including more than 1000 species [6] in response to harsh environments. Spores can survive in a dormant state for long periods, resisting stresses such as high temperature, dehydration, absence of nutrients, and presence of toxic chemicals. Their use as a drug/antigen delivery system has been fostered by their high stability [7] and by the safety record of several species of spore formers [8]. Initially, spores of the model organism *Bacillus subtilis* were used [9], but other *Bacillus* species have also been tested for the display and mucosal delivery of antigens and enzymes [4,10].

Here, the use of spores of *B. megaterium* for the delivery of therapeutic molecules to the gastrointestinal mucosa is proposed. The *B. megaterium* spore is particularly promising as a delivery vehicle due to its large dimensions (length up to 3 μm and diameter of 1 μm, with a spore surface area about 2.5-fold larger than that of *B. subtilis*) [11] and the presence of an exosporium, a protective layer surrounding the spore found only in some spore-forming species [12,13]. The exosporium of

Abbreviations: Bcp1, bacterioferritin comigratory protein 1; mRFP, red fluorescent protein of the coral *Discosoma* sp; DSM, Difco Sporulation mdium; LB, Luria-Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; EDTA, Ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DTT, DL-Dithiothreitol; FITC, fluorescein isothiocyanate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; BSA, bovine serum albumin; PBS, phosphate buffered saline

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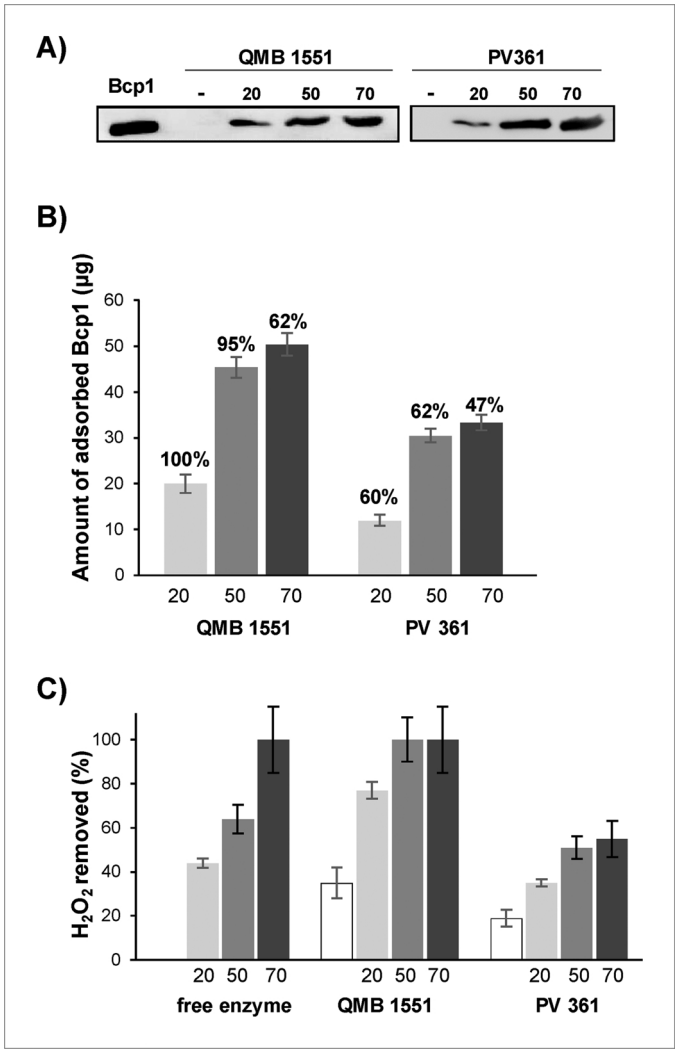

Fig. 1. Adsorption of Bcp1 to *B. megaterium* spores.
5×10^8 spores of QM B1551 and PV361 strains were incubated with 20, 50 or 70 µg of Bcp1 and then the samples subject to centrifugation. (A) Spore surface proteins were extracted from the pellet fractions by SDS-DTT treatment, separated by SDS-PAGE and analyzed by western blot with an anti-polyhistidine antibody conjugated to horseradish peroxidase. Free Bcp1 (Bcp1) was used as a marker and free spore protein extracts as control (-). Whole images of western blots are reported in the Supplementary material. (B) The percentage of spore-adsorbed Bcp1 was calculated from dot blotting (Figs. 1 and 2) of the supernatants fractions containing unbound Bcp1, and relative densitometric analysis (Supplementary material, Additional Table 1–2). (C) Peroxidase activity of QM B1551 spores (grey bars), of 20 and 50 µg of free Bcp1 (white bars) or of the same amounts of enzyme adsorbed to spores (black bars). *Error bars* show the standard errors of the mean from the three different experiments. P value < 0.05.

spores of QM B1551, the best-characterized strain of *B. megaterium*, have recently been shown to be essential for the adsorption of the red fluorescent protein of the coral *Discosoma* sp (mRFP) [14]. mRFP was shown to cross the exosporium and localize in the inter-coat space [14].

We used a well-characterized archaeal enzyme, the bacterioferritin comigratory protein 1 (Bcp1) of *Sulfolobus solfataricus*, belonging to peroxiredoxin family [15–19], as a model to study the spore-based delivery of therapeutic agents to mucosal surfaces. Peroxiredoxins are thiol peroxidases commonly found in archaea and eukaryotes, including humans, and known to contribute to cell protection against inorganic and organic peroxides [18]. Recently, the highly thermostable Bcp1 of *S. solfataricus* has been found to protect cardiomyoblasts from oxidative stress *in vitro* and was proposed as potentially a health beneficial molecule with anti-oxidant activity [20]. The delivery of enzymes with antioxidant activity, such as Bcp1, may be a new strategy to address inflammation caused by oxidative stress [21,22].

Materials and methods

Materials and suppliers

Difco Sporulation medium (DSM) - Oxoid, UK, 234000; BRAND® counting chamber BLAUBRAND® Bürker-Türk-Sigma, USA, BR719505; Isopropyl-β-D-thiogalactoside (IPTG) - Sigma, USA,6758; Ethylenediaminetetraacetic acid (EDTA) - Sigma, USA, E9884; Protran 0.45 NC nitrocellulose Western blotting membranes - Amersham Pharmacia Biotech, 10600002; His Trap HP – GE Healthcare, USA, 11-0012-38 AH; DL-Dithiothreitol (DTT) - Sigma, USA, D0632; Anti-polyhistidine-horseradish peroxidase coupled antibody - Sigma, USA, A7058; Bovine serum albumin (BSA) - Sigma, USA, A2153

Bacterial strains and spore purification

B. megaterium strains QM B1551 and PV361 [13] and *B. subtilis* strain PY79 [23] were used. Sporulation was induced by the exhaustion method [8]. After 30 h growth in Difco Sporulation medium (DSM) at 37 °C with vigorous shaking, spores were collected, washed 3 times with distilled water and incubated overnight in distilled water at 4 °C to lyse residual sporangial cells as previously described [14]. Spore counts were determined by direct counting with a Bürker chamber [Sigma, USA (BR719505)] under an optical microscope (Olympus BH-2 with 40× lens).

Expression and purification of Bcp1

BL21 *Escherichia coli* strain expressing Bcp1 protein [16] was grown to 0.8 OD_{600nm} in Luria-Bertani (LB) medium supplemented with kanamycin (10 µg mL-1) and chloramphenicol (34 µg mL-1) at 37 °C. Expression was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG) [Sigma, USA (6758)] for 6 h at 37 °C as previously described [16,24]. The cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl pH 8.0 containing a complete EDTA [Sigma, USA (E9884)] free

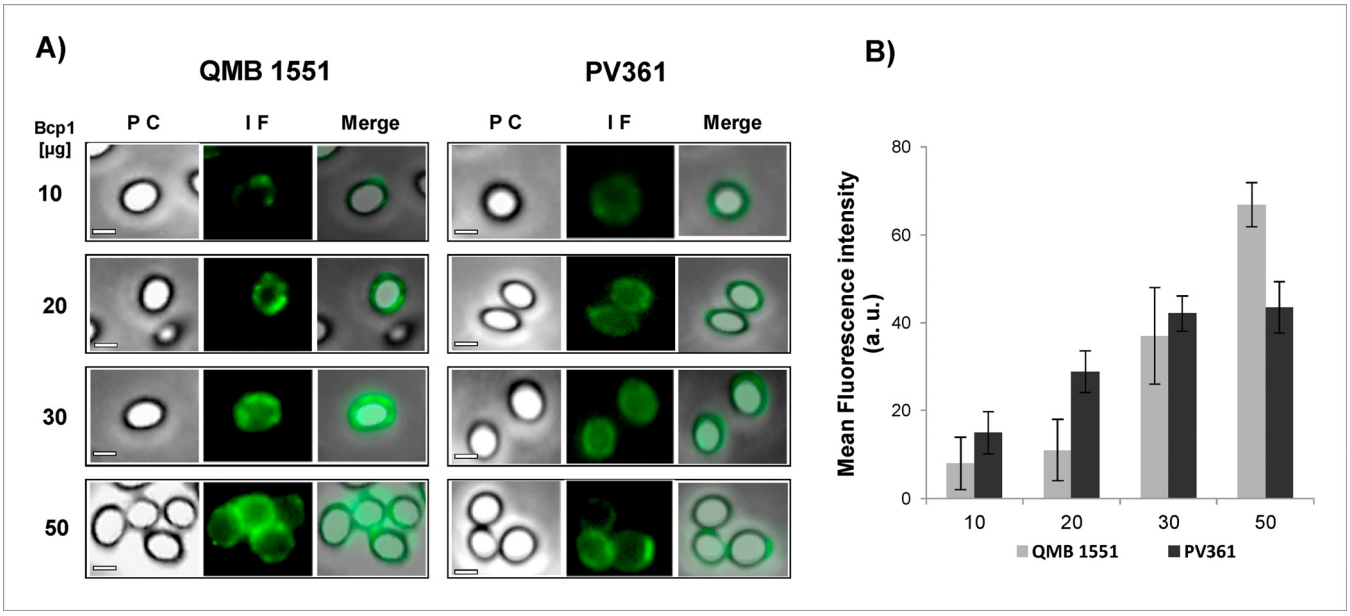

Fig. 2. Immunofluorescence microscopy of *B. megaterium* spores adsorbed with increasing amounts of purified Bcp1. (A) After adsorption reaction with different amount of Bcp1 (10, 20, 30, or 50 µg) of Bcp1, QMB 1551 and PV361 spores were reacted with mouse anti-polyhistidine primary antibody and fluorescein isothiocyanate-conjugated secondary anti-mouse IgG. The same microscopy field for phase contrast (PC) and immunofluorescence (IF) is shown together with the merge panel. The exposure time was 500 ms for all images. Scalebar,1 µm. (B) Data are expressed as fluorescence intensity in arbitrary unit (a.u.) and represent the mean of fifty spores in each group. P value < 0.01.

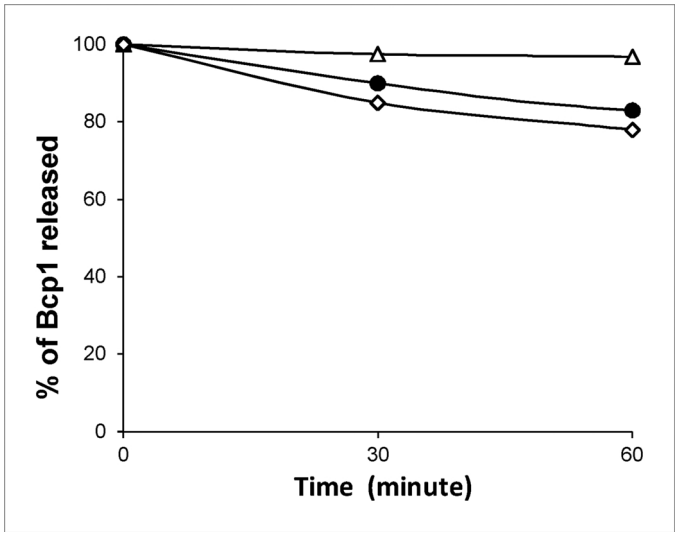

Fig. 3. Kinetics of Bcp1 release. Bcp1-adsorbed spores were incubated either in 0.1 M glycine – HCl, pH 2.0 (triangles), 0.1 M citrate-phosphate pH 6.0 (circle) or 0.1 M HEPES pH 7.0 (diamonds) and the amount of released Bcp1 estimated by dot blot assay and its relative densitometric analysis (Supplementary material, Additional Fig. 3).

protease inhibitors cocktail and disrupted by ultrasonication with 20 min pulses at 20 Hz (Sonicator Ultrasonic liquid processor; Heat System Ultrasonics Inc.).

Protein purification was carried out in two steps: heat treatment of the cell extract at 80 °C for 15 min and affinity chromatography by His Trap HP [19]. The suspension was clarified by ultracentrifugation at 160,000 x g for 30 min. The crude extract obtained was heated at 80 °C for 15 min, and then centrifugated at 15,000 x g at 4 °C for 30 min removing almost 70% of the mesophilic host proteins. The extract was concentrated (Amicon, Millipore Corp.; Bedford, MA, USA) and applied to a HisTrap HP [GE Healthcare, USA (1-0012-38 AH)] equilibrated with 50 mM Tris/HCl, pH 8.0, 0.3 M NaCl (buffer A). The column was washed with buffer A with 20 mM imidazole, and proteins were eluted with buffer A supplemented with 250 mM imidazole. The active fractions were pooled and dialyzed against 20 mM Tris/HCl, pH 8.0. [16].

Adsorption reaction

Different amounts of purified Bcp1 were incubated with 5×10^8 spores in 200 µl of 50 mM sodium citrate pH 4.0 at 25 °C [25,26]. After 1 h incubation, the binding mixture was centrifuged (10 min at 13,000xg at room temperature) to separate Bcp1-adsorbed- spores in the pellet from free Bcp1 in the supernatant [25,26].

Western blot and dot-blot analysis

Extraction of proteins from spores and Bcp1-adsorbed spores was performed by treatment at 65 °C in 40 µl of sodium dodecyl sulfate (SDS)-dithiothreitol (DTT) extraction buffer [10 mM tris pH 8.0, 1% SDS, 50 mM DTT, 10 mM EDTA, 50 mM Tris – HCl, pH 8.0]. 20 µl of extracted proteins were separated on 12.5% denaturing poly- acrylamide gels, electrotransferred to nitrocellulose membranes [Amersham

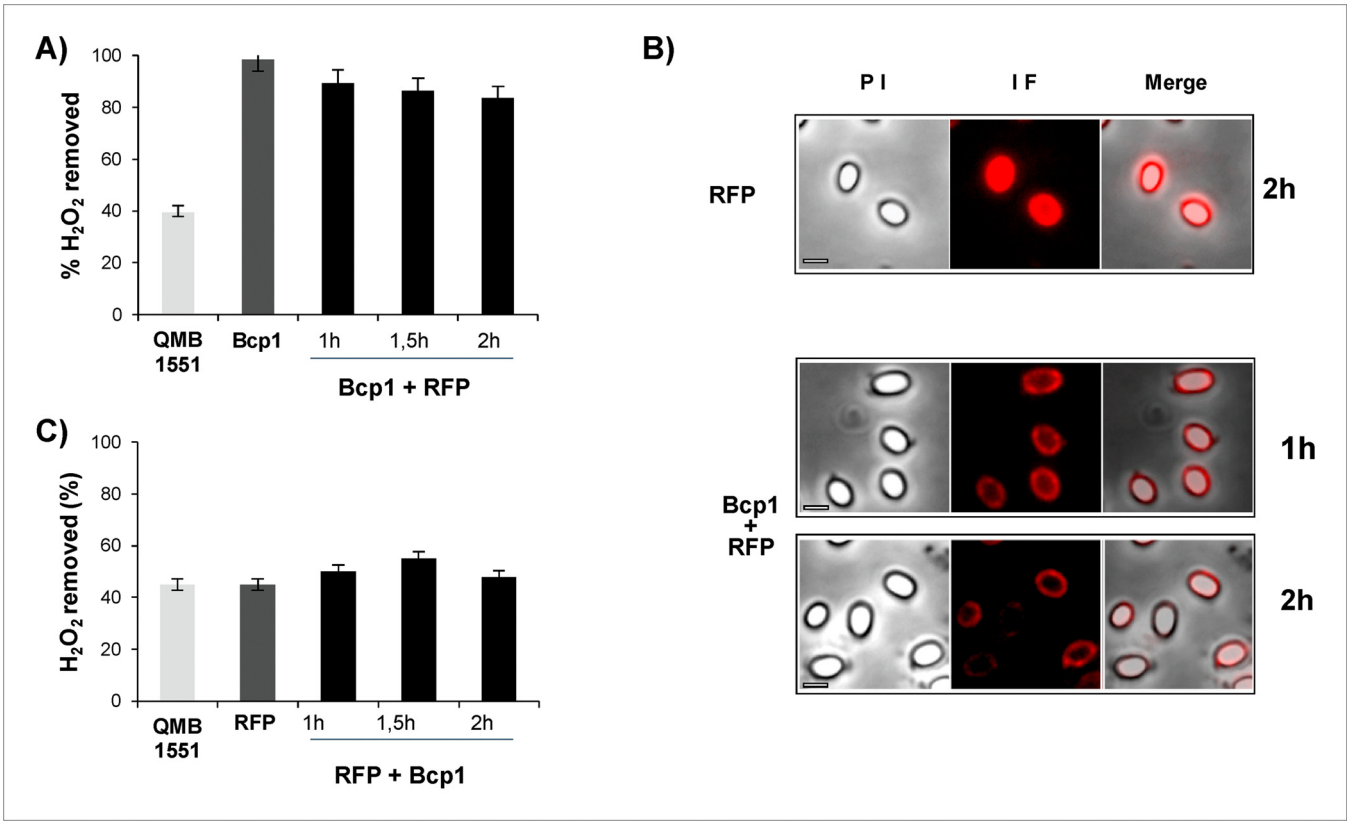

Fig. 4. Displacement assays.
(A) Peroxidase activity of 5.0×10^8 spores of QMB 1551 alone (light grey bar) or adsorbed with 50 µg of Bcp1 (Bcp1 - dark grey bar) and then with mRFP (Bcp1 + RFP - black bars)., The assay was performed after 1, 1.5 and 2 h from the addition of mRFP. (B) QM B1551 spores were adsorbed with 5 µg of mRFP (RFP) or with 50 µg of Bcp1 and then with 50 µg of mRFP (RFP + Bcp1), washed and analyzed by fluorescence microscopy after 1 or 2 h of incubation. The livery of enzymes with antioxidant activity, such as Bcp1, may be a same field was observed by phase contrast and fluorescence microscopy. The merge panel is shown. The exposure time was 200 ms. Scalebar,1 µm. (C) Peroxidase activity of QMB 1551 spores (light grey bar) or adsorbed with 50 g of mRFP (RFP - dark grey bar) and then with 50 g of Bcp1 (RFP + Bcp1 - black bars); the experiment was performed as described in (A).

Pharmacia Biotech, (10600002)] and analysed by western blot using a monoclonal anti-polyhistidine-horseradish peroxidase coupled antibody (1:7000) [Sigma, USA (A7058)], as previously reported [26]. A quantitative determination of the amount of Bcp1 was obtained by dot blot experiments comparing serial dilutions of purified Bcp1 and binding assay supernatant. Filters were then visualized by the ECL-substrates method (Clarity, Bio-rad) and subjected to densitometric analysis by Quantity One 1-D Analysis Software (Bio-Rad) [26]. Dot blot and relative densitometric analyses were performed 3 times.

Fluorescence and immunofluorescence microscopy

Immunofluorescence experiments were performed as described [14]. 2.0×10^6 Bcp1-adsorbed spores of QM B1551 and PV361 were incubated with 1% bovine serum albumin (BSA) ([Sigma, USA (A7058)] for 30 min and then for 2 h with mouse monoclonal anti-polyhistidine antibodies (1:200) in 1xPBS-1% BSA. Samples were washed and treated with a 1:64 goat anti-mouse IgG secondary antibody conjugated with fluorescein isothiocyanate, FITC [Thermo Fisher Scientific, USA (62-6511)] (1 h at 4 °C, in the dark). After 4 washes, the samples were resuspended in 20 µl of 1xPBS and 10 µl were analyzed.

For the analysis of mRFP-adsorbed spores, following the adsorption reaction the spores were resuspended in 50 µl of 1xPBS pH 4.0 and 5 µl of the suspension observed by fluorescence microscopy [27].

All samples were observed with an Olympus BX51- DP70 fluorescence microscope fitted with a 100x UPlan F1 oil objective; U-MNG or U-MWIBBP cube-filters were used to detect the red fluorescence emission of mRFP or the green emission of FITC-conjugated antibodies respectively. ImageJ (v1.48, NIH) was used to draw an outline around 50 spores for each strain and mean fluorescence values per pixel were recorded for each spore [14,27].

Peroxidase activity

The peroxidase activity of free or spore-adsorbed Bcp1 was tested as previously reported [28]. 100 µl of reaction mixture (50 mM HEPES pH 7.0, 10 mM DTT 0.2 mM H_2O_2) containing different concentrations of Bcp1, spores or Bcp1-adsorbed spores were incubated at 37 °C for 5 min. 900 µl of trichloroacetic acid (10%, w/v) were added to stop the reactions and the mixture was combined with 200 µl of 10 mM ferrous ammonium sulfate $Fe(NH_4)_2(SO_4)_2$ and 100 µl of 2.5 M potassium thiocyanate KSCN, which led to the formation of a red colored iron complex. The peroxide concentration is proportional to the color intensity measured at $\lambda = 492$ nm [28]. The percentage of peroxide removed was calculated on the basis of the change in A_{492nm} obtained with Bcp1 relative to that obtained without Bcp1 [28].

Treatments with simulated gastric and intestinal fluids

Free Bcp1, spores and Bcp1-adsorbed spores were incubated for 1 h at 37° in 100 µl of simulated gastric juice (SGF) [1 mg of pepsin (porcine stomach mucosa; Sigma) per ml of 10 mM HCl; pH 2.0] or small intestine fluid (SIF) [1 mg of pancreatin (porcine pancreas; Sigma) per ml and 0.2% bile salts (50% sodium cholate-50% sodium deoxycholate; Sigma); pH 6.8]. To remove the proteases contained in SIF and SGF, after incubation, the samples containing free Bcp1 were treated at 90 °C

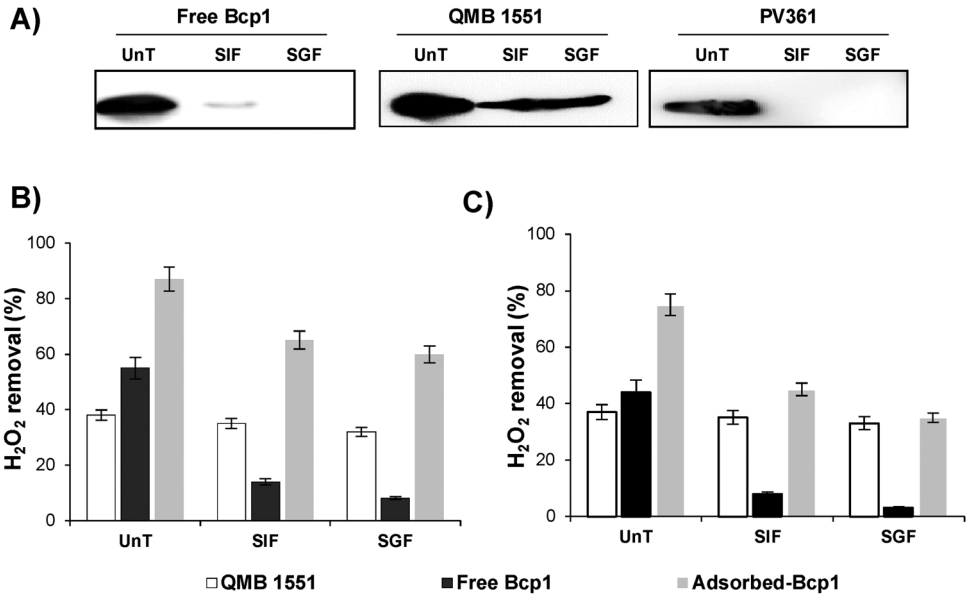

Fig. 5. Effect of SIF and SGF on spore adsorption. (A) Western blotting, performed with Bcp1-recognizing anti-polyhistidine antibody, of free Bcp1 and of proteins extracted from spores of *B. megaterium* QM B1551 and PV361 adsorbed with 30 μg and 50 μg of Bcp1 respectively. Free and spore-adsorbed enzyme was not treated (UnT) or treated with SIF or SGF (see Methods). Whole images of western blots are reported in the Supplementary material. (B) Peroxidase activity of 5.0×10^8 spores of QM B1551 (white bars), of 50 μg of free Bcp1 (black bars) or of the same amount of enzyme adsorbed to spores (grey bars) without (UnT) or with SIF or SGF treatment. (C) The experiment was performed as described in (B), but using 20 μg of enzyme. Error bars show the standard errors of the mean of three independent experiments ($P < 0.05$).

for 15 min while samples containing spores were centrifuged for 10 min at 13000xg [29].

For the pH-stability assay, free Bcp1, spores and Bcp1-adsorbed spores were incubated at 37 °C for 1 h in following buffers: 0.1 M glycine-HCl pH 2.0; 0.1 M citrate-phosphate pH 4.0 and pH 6.0, or 0.1 M HEPES pH 7.0. After incubation, the peroxidase activity of the samples was measured following the protocol described above [16].

Statistical analysis

Results of peroxidase activity analysis are the means of 3 independent experiments. The error bars reported in the figures show the standard errors of the mean from the 3 experiments. Statistical significance was determined by Student t-test, using Microsoft Office Excel, and the significance level was set at $P < 0.05$.

Results and discussion

Display of active Bcp1 of S. solfataricus on B. megaterium spores

To verify whether *B. megaterium* spores were able to adsorb Bcp1 onto their surface, 20, 50 or 70 μg of the purified enzyme was incubated with 5.0×10^8 purified spores of QM B1551 or PV361. The reactions were performed in 50 mM sodium citrate at pH 4.0 [25]. After the reaction, spores were extensively washed with PBS pH 4.0, collected by centrifugation, surface proteins extracted and analyzed by western blotting. As shown in Fig. 1A, specific signals were observed with extracts of spores reacted with the different amounts of purified Bcp1, indicating that Bcp1 was absorbed during the reaction by spores of both strains and released by the extraction treatment.

Bcp1 was apparently extracted in comparable amounts from spores of QM B1551 previously adsorbed with 50 and 70 μg of the enzyme. To confirm this observation, a well-established procedure [9,14,26,27] was followed and the amount of Bcp1 remaining unbound was quantified, *i.e.*, post-adsorption spores were collected by centrifugation and the supernatant serially diluted and analyzed by dot blotting (Supplementary material, Additional Figs. 1 and 2). The results of the densitometric analysis (Supplementary material, Additional Tables 1 and 2) are shown in Fig. 1B and indicated that with QMB 1551 spores, almost all the Bcp1 was adsorbed when using 20 or 50 μg in the reaction (100 and 95% respectively). The efficiency of adsorption decreased by about 40% when 70 μg of enzyme was used, suggesting saturation of the adsorption with 50 μg (Fig. 1B). With PV361 spores, the efficiency of adsorption was lower than with QMB 1551 at all concentrations of Bcp1. With the lowest Bcp1 concentration analyzed (20 μg), only 60% of the enzyme was adsorbed and a maximum of about 30 μg of adsorbed Bcp1 was observed when 50 or 70 μg was used in the reaction (Fig. 1B). Based on these results, it was concluded that 5.0×10^8 spores of QMB 1551 are able to adsorb about 50 μg and that the exosporium, present in QM B1551 and absent in PV361, is essential for an efficient adsorption of Bcp1.

To assess whether spore-adsorbed Bcp1 retained its enzymatic activity the efficiency of H_2O_2 removal by the free and spore-bound enzyme was assayed. As a control, *B. megaterium* QM B1551 and PV361 spores alone were assayed and showed an antioxidant activity (white bars in Fig. 1C). Interestingly, PV361 spores were less efficient than QM B1551 in removing H_2O_2, suggesting that antioxidant activity was localized in the exosporium. Although we cannot distinguish between the enzymatic activity due to the adsorbed Bcp1 or to the spore itself, QM B1551 spores adsorbed with Bcp1 had an antioxidant activity higher than similar amounts of free Bcp1 and also than spores alone, indicating that the adsorbed enzyme was active (grey bars in Fig. 1C). With Bcp1-adsorbed PV361 spores, the antioxidant activity was lower than the free enzyme but higher than that of spores alone, suggesting that the small amount of enzyme adsorbed to spores (Fig. 1B) was active (Fig. 1C). Thus, the activity observed with Bcp1 adsorbed to spores is most likely due to the combination of the activities of the adsorbed enzyme and of the spores, with QM B1551 spores being more efficient that PV361 in

adsorbing active Bcp1.

Bcp1 localizes in the inter-coat space

Immunofluorescence was used to localize the adsorbed Bcp1 on the spore surface. 5.0×10^8 spores of strains QM B1551 or PV361 after adsorption of various amounts of Bcp1, were reacted with Bcp1-recognizing anti-polyhistidine antibody and a fluorescent secondary anti-mouse IgG. As shown in Fig. 2A, with QMB 1551 spores almost no fluorescence was observed when 10 µg of Bcp1 was used and only a weak fluorescence signal, localized in spots, was observed with 20 µg of enzyme. Stronger signals were observed by increasing the amount of Bcp1 to 30 and 50 µg; however, with 30 µg the signal appeared non-uniform but rather comprised of spots interrupted by non-fluorescent regions (Fig. 2A). With PV361 spores, the fluorescent signal was almost absent with 10 µg of enzyme but present and evenly distributed around the spores with all other concentration of Bcp1 (Fig. 2A).

The fluorescent signal intensity was quantified using ImageJ software [11]. The mean value determined was higher with PV361 than with QMB 1551 spores when 10 or 20 µg Bcp1 were used (Fig. 2B). The signals were of similar intensity when spores of the two strains were reacted with 30 µg and became stronger with QM B1551 than PV361 when 50 µg of enzyme were used (Fig. 2B). These results are not consistent with the previous results (Fig. 1) showing that QM B1551 were more efficient than PV361 spores in absorbing Bcp1.

To explain this apparent inconsistency, we hypothesized that, with QM B1551 spores, the enzyme was unavailable to the Bcp1-detecting antibody because the Bcp1 had crossed the exosporium and localized in the inter-coat space, as previously reported for the mRFP protein [11]. According to this, when 10 or 20 µg of Bcp1 were used for the adsorption reaction all Bcp1 crossed the exosporium of QMB 1551 spores, localizing in the inter-coat space and becoming mostly unavailable to antibody. By increasing the amount of Bcp1 in the adsorption reaction, the inter-coat space was gradually filled up and with 30 and 50 µg of enzyme strong but still irregular signals were observed (Fig. 2A). With spores lacking the exosporium (PV361) all Bcp1 adsorbed to spores was recognized by antibody, producing a strong and uniformly distributed signal.

Bcp1 is stably and tightly adsorbed to QM B1551 spores

To test the stability of Bcp1 adsorption, adsorbed spores were incubated at 37 °C under various conditions of pH. After 30 and 60 min, spores were collected by centrifugation and the amount of Bcp1 present in the supernatant measured by dot-blotting (Supplementary material, Additional Fig. 3). Fig. 3 shows the results of the densitometric analysis of the dot-blot (Supplementary material, Additional Table 3) and indicated that at pH 4.0 no Bcp1 was found in the supernatants, while at pH 6 and 7 some of the adsorbed enzyme was released. The release of a maximum of 20% of Bcp1 after 1 h at pH 7.0 was unsurprising, as it was already known that the adsorption of several other proteins to spores of *B. subtilis* or *B. megaterium* preferentially occurred at pH 4.0 and was less efficient at other pHs [13,26,27].

To further analyze the stability of the interaction between Bcp1 and QM B1551 spores a displacement assay was performed using *Discosoma* red fluorescent protein mRFP, already shown to efficiently adsorb onto QM B1551 spores [14]. 5×10^8 spores were reacted with 50 µg of Bcp1, washed and then incubated with 50 µg of mRFP. After 1, 1.5 and 2 h incubation, the antioxidant activity of spores was measured. As shown in Fig. 4A, antioxidant activity measured in the absence of mRFP (dark grey bar) was only slightly reduced by incubation with the competing protein (black bars), suggesting that the adsorbed Bcp1 was not displaced by mRFP. Spores adsorbed with Bcp1 and then with mRFP were also analyzed by fluorescence microscopy. As shown in Fig. 4B, the red fluorescence signal was weak compared to spores adsorbed only with mRFP and did not increase over time, confirming that mRFP was not able to replace the previously adsorbed Bcp1. In a parallel experiment, spores were reacted with mRFP and then with Bcp1. Consistently, the antioxidant activity of spores was only minimally increased by the adsorption of Bcp1 (Fig. 4C, black bars), indicating that Bcp1 was also unable to displace mRFP. The results indicate that the adsorption of heterologous proteins to spores is tight and cannot be displaced by a second heterologous protein present in high concentration outside the spore.

Effects of simulated gastric or intestinal conditions on Bcp1-adsorbed spores

To analyze the effects of simulated intestinal conditions on Bcp1 adsorbed onto spores, Bcp1-adsorbed spores of the QMB 1551 and PV361 strains were treated with simulated gastric fluid (SGF) or simulated intestinal fluid (SIF). Since spores of the two strains adsorb Bcp1 with different efficiencies (Fig. 1), in order to have similar amounts of enzyme adsorbed to spores of the two strains, 5.0×10^8 spores of the QMB 1551 and PV361 strains were adsorbed with 30 µg and 50 µg respectively. After the adsorption reaction, spores were treated with SGF or SIF, washed, and used to extract surface proteins which were analyzed by western blotting as above. In parallel, the same amount of free Bcp1 was also treated with SGF or SIF and analyzed by western blot. As shown in Fig. 5A, free Bcp1 was totally degraded by SGF treatment and only a minimal amount of the enzyme was still detected after the SIF treatment. Bcp1 adsorbed to PV361 spores, lacking the exosporium, was totally degraded by both SGF or SIF treatments. Bcp1 molecules were, in contrast, still extractable and detected after either treatment when adsorbed on QMB1551 spores, indicating that they were only partially affected by SGF or SIF (Fig. 5A).

To verify whether the enzyme activity was still present on QMB 1551 spores after the SIF or SGF treatment, their antioxidant activity was analyzed. As shown in Fig. 5B, the antioxidant activity of the spores alone was not affected by either treatment (white bars), while the activity of the free enzyme (50 µg) was strongly affected by both (black bars). The activity of 5.0×10^8 spores adsorbed with 50 µg of Bcp1 was also affected, but the reduction was about 20% and 30% with SIF and SGF respectively (Fig. 5B). Both SGF and SIF affected free and spore-bound Bcp1, but the antioxidant activity of spore-bound Bcp1 was slightly higher than the sum of the activities of spores and free enzyme (Fig. 5B), suggesting that at least part of the adsorbed enzyme was still active after the treatments. Partial protection from degradation of the adsorbed Bcp1 is not surprising, since it has been previously reported that the β-galactosidase of *Alicyclobacillus acidocaldarius* was protected against heat and acidic conditions when adsorbed on *B. subtilis* spores [25].

For Bcp1, protection was only observed with QM B1551 spores and not with PV361 (Fig. 5A), indicating that the exosporium is essential for this effect. A possible explanation for the partial protection of Bcp1 on QM B1551 is that the enzyme, localized in the inter-coat space (Fig. 2), was protected by the exosporium and was not accessible to degradative enzymes. We hypothesized that these proteases were not able to cross the exosporium and degrade Bcp1 because, as shown for mRFP (Fig. 4), the inter-coat space was already fully occupied by Bcp1. For verification, the experiment of Fig. 5B was repeated, adsorbing QM B1551 spores with a reduced amount of enzyme (20 µg), insufficient to completely fill the inter-coat space. As shown in Fig. 5C, the activity of spores adsorbed with 20 µg of Bcp1 was strongly reduced by SIF and almost completely eliminated by the SGF. The antioxidant activity of spore-bound Bcp1 was slightly higher than or identical to that of spores alone after the SIF or SGF treatment, respectively (Fig. 5C), indicating that the enzyme was almost completely degraded. Thus, under these conditions, some of the protease contained in SGF and SIF apparently crossed the exosporium and degraded Bcp1, supporting the hypothesis that the protection of Bcp1 observed in Fig. 5A, B was due to the inability of the degradative molecules to enter the pre-filled inter-coat space.

Conclusions

The main conclusion of this report is that the thermoacidophilic enzyme Bcp1 of *S. solfataricus* can be efficiently adsorbed nto spores of *B. megaterium*, with about 50 μg adsorbed by 5.0×10^8 spores. Based on the deduced molecular mass of 17.46 kDa for Bcp1, we estimated that about 9.5×10^9 molecules of Bcp1 can be displayed on each spore. The adsorption is in part due to the presence of the exosporium. Adsorbed Bcp1 localized in the space between the outer coat and the exosporium and this unusual localization contributed to the protection of the enzyme from degradation by treatment with SIF or SGF.

The Bcp1-spore interaction is highly stable. The enzyme was not spontaneously released under the reaction conditions and the presence of high concentrations of other molecules outside the spore did not displace the adsorbed molecules. However, a partial release of the adsorbed Bcp1 was observed when the pH conditions shifted from acidic to neutral. All these properties are particularly desirable for an oral delivery system aimed at crossing the stomach and delivering the transported molecules at the level of the intestinal mucosal surfaces.

An additional interesting observation highlighted by this report is that *B. megaterium* spores have an endogenous antioxidant activity. This is in part associated with the exosporium and represents a potentially useful property for a display platform to be used for the delivery of molecules to animal mucosal surfaces.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MML - performed most of the experiments; GD - contributed to spore production and purification and enzymatic assays; FP and CS-contributed to enzyme purification and enzymatic assays; DL - contributed to experiment design; ER - contributed discussions and suggestions; RI - contributed to experiment design and writing of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2018.06.004.

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