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Gut Adhesive *Bacillus subtilis* Spores as a Platform for Mucosal Delivery of Antigens

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***Bacillus subtilis* spores have been used as safe and heat-resistant antigen delivery vectors. Nonetheless, the oral administration of spores typically induces weak immune responses to the passenger antigens, which may be attributed to the fast transit through the gastrointestinal tract. To overcome this limitation, we have developed *B. subtilis* spores capable of binding to the gut epithelium by means of expressing bacterial adhesins on the spore surface. The resulting spores bound to *in vitro* intestinal cells, showed a longer transit through the mouse intestinal tract, and interacted with Peyer's patch cells. The adhesive spores increased the systemic and secreted antibody responses to the *Streptococcus mutans* P1 protein, used as a model antigen, following oral, intranasal, and sublingual administration. Additionally, P1-specific antibodies efficiently inhibited the adhesion of the oral pathogen *Streptococcus mutans* to abiotic surfaces. These results support the use of gut-colonizing *B. subtilis* spores as a new platform for the mucosal delivery of vaccine antigens.**

Bacterial carriers for the mucosal delivery of antigens, particularly those capable of colonizing or invading through the mucosal epithelia, have been intensively investigated (1). Live bacterial vectors can be generated with attenuated pathogens, such as *Salmonella* and *Listeria*, or nonpathogenic commensal species, such as *Lactococcus* and *Lactobacillus*. Bacteria in the former group of are capable of inducing strong and long-lasting immune responses to passenger antigens but present serious safety concerns, whereas bacteria in the latter group are safer but typically induce much lower immune responses to the passenger antigens (1–3). As a safe nonpathogenic live bacterial vector, *Bacillus subtilis*, a spore-forming soil Gram-positive bacterial species, has been engineered to express antigens as either vegetative cells or spores (4, 5). As antigen carriers, *B. subtilis* spores have several attractive features, including a safe record of human and animal use as both probiotic and food additives, remarkable heat resistance, and rather easy genetic and bacteriological manipulation.

Currently, two major genetic approaches have been proposed to generate recombinant *B. subtilis* spores as vaccine delivery vectors. The first approach relies on the expression of a heterologous protein genetically fused to surface-exposed spore coat proteins, such as CotB, CotC, or CotG (6, 7). Such a strategy would allow a better presentation of the passenger antigen to the mucosa-associated lymphoid tissue (MALT) afferent sites, leading to the induction of adaptive immune responses, such as mucosal secretory (IgA) or systemic (IgG) antigen-specific antibody responses (6–8). The second approach is based on a distinct rationale and has employed episomal vectors in which the target antigen is expressed under the control of a promoter (P*gsiB*) active only at the vegetative cell stage, which means immediately after spore germination (9–11). This antigen delivery approach relies on the fact that *B. subtilis* spores germinate during transit through the gastrointestinal tract and produce the target antigen at the intestinal lumen or inside the phagocytes of antigen-presenting cells (APCs), leading to the induction of antibody responses in the serum (IgG) and mucosa (fecal IgA) (9–11).

However, in both cases, the administration of recombinant spores via mucosal routes typically confers immune responses to the passenger antigen lower than those achieved with delivery systems based on attenuated bacterial strains capable of colonizing the mammalian gastrointestinal tract. The reduced mucosal adjuvant effects of *B. subtilis* spores have been attributed to several factors, such as a previously established immunity generated by the frequent ingestion of spores, the reduced amount of expressed antigens, and the rapid transit through the gastrointestinal tract, which reduces the chances of a productive interaction with the gut-associated lymphoid tissue (GALT), such as M cells and APCs at Peyer's patches (PPs) (12, 13).

In an attempt to improve the performance of *B. subtilis* cells as antigen delivery vectors following mucosal spore administration, we combined the two different protein expression approaches. First, spores were engineered to express bacterial adhesins at the spore surface by genetic fusion with CotB, a *B. subtilis* spore coat protein. Two previously known bacterial adhesins known to promote the colonization of the mammalian gastrointestinal tract were employed: the S-layer protein (SlpA) from *Lactobacillus brevis* and invasin (InvA) expressed by *Yersinia pseudotuberculosis* (14, 15). In a second step, the spores were genetically modified to express at the cell stage a protein fragment (the P1 protein fragment from amino acids 39 to 512 [$P1_{39\text{-}512}$]) derived from the P1 antigen (also known as antigen I/II, Pac, or antigen B) and originally expressed by *Streptococcus mutans*, the causative agent of

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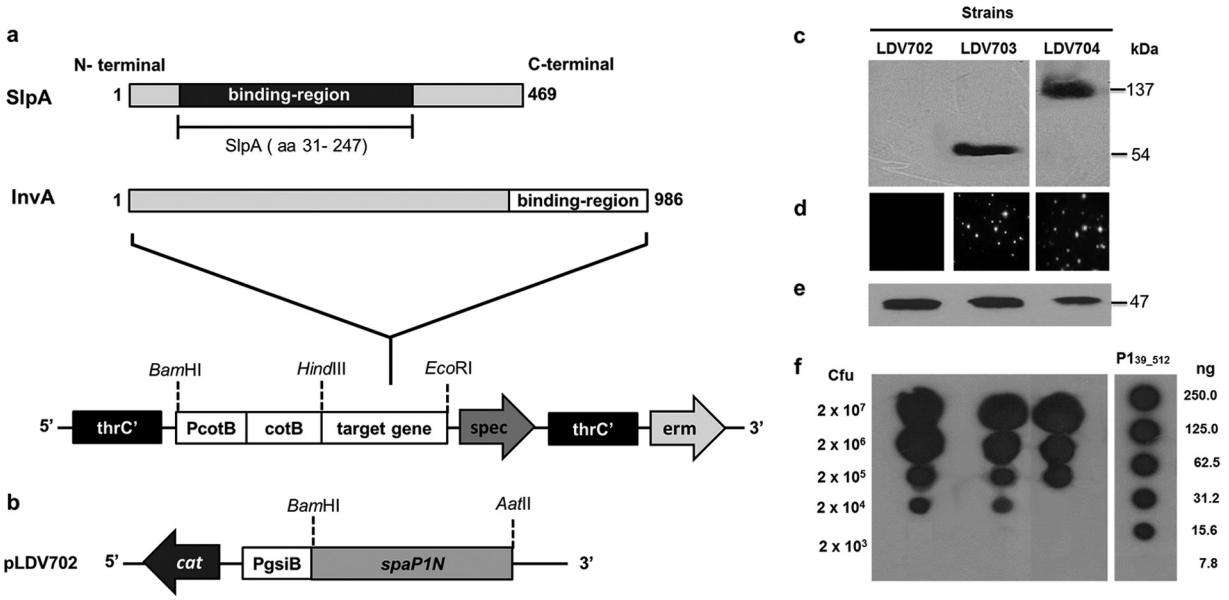

FIG 1 Generation of recombinant *B. subtilis* spores that have adhesins at the spore surface and that encode a target antigen after spore germination. (a) Cloning strategies used to generate the expression vectors employed in the present study. The pLDV703 and pLDV704 plasmids encode the SlpA-binding region of *L. brevis* and *Y. pseudotuberculosis* InvA, respectively, genetically fused to the N-terminal region of CotB, allowing the anchoring of the recombinant proteins at the outer spore coat. aa, amino acids. (b) The pLDV702 vector encodes *S. mutans* $P1_{39-512}$ as an intracellular antigen under the control of the P*gsiB* promoter. (c) Expression of SlpA and InvA adhesins by recombinant *B. subtilis* spores. The spore coat proteins of LDV703 and LDV704 strains were sorted by SDS-PAGE and reacted with anti-SlpA- and anti-InvA-specific polyclonal antibodies. (d) Surface display of SlpA and InvA by recombinant *B. subtilis* spores monitored by immunofluorescence. Purified spores were reacted with antibodies specific for SlpA (LDV703) or InvA (LDV704). LDV702 spores, used as a negative control, were incubated with both polyclonal antibodies. Magnification, ×1,000. (e) Expression of the *S. mutans* $P1_{39-512}$ antigen by *B. subtilis* vegetative cells. *B. subtilis* cells were submitted to a temperature shift, and $P1_{39-512}$ expression was monitored by Western blotting in which the blots were developed with P1-specific sera. (f) Quantification of $P1_{39-512}$ expression by the recombinant *B. subtilis* strains by dot blotting. The cells were blotted on nitrocellulose sheets and reacted with P1-specific serum. *B. subtilis* strain LDV702 was used as a reference. Nonspecific reactions with anti-P1 were not detected with *B. subtilis* strain 1012. Known concentrations of the purified recombinant $P1_{39-512}$ protein were used to determine the concentrations of the proteins expressed by each recombinant *B. subtilis* strain. The molecular masses of the recombinant proteins are indicated on the right side of panels c to e.

dental caries, but also found on the surface of different pathogenic streptococci (16–18). Notably, the *S. mutans* P1 protein represents the major antigen target for most anticaries vaccine approaches previously reported (19).

Our findings demonstrate that engineered *B. subtilis* spores expressing bacterial adhesins persisted longer in the mouse gastrointestinal tract, particularly at Peyer's patches, and enhanced the antibody responses to the passenger antigen following delivery via the oral, nasal, and sublingual routes. Altogether, the reported strategy represents a new antigen delivery platform based on *B. subtilis* spores.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5α and *B. subtilis* 1012 (20) strains were routinely cultivated aerobically in Luria-Bertani (LB) broth (37°C). *S. mutans* strain NG8 (kindly provided by L. Jeannine Brady, University of Florida) was cultivated in Todd-Hewitt broth and yeast extract (0.3%) (THY) at 37°C in 5% CO_2. Antibiotics were added to the growth media according to the strain and plasmid used. Competent *E. coli* and *B. subtilis* cells were used following established procedures (21, 22).

Construction of the recombinant *B. subtilis* strains. The genes encoding the full-length *Y. pseudotuberculosis* InvA, which interacts with the β1 integrin receptor and promotes the invasion of gut epithelial cells (23–25), and the *L. brevis* S-layer protein (SlpA), directly involved in the binding to fibronectin and conferring the capability to colonize the mammalian gut (26, 27), were fused to the gene encoding the N-terminal portion of the CotB protein (275 amino acids), which has previously been employed to expose different heterologous proteins at the spore surface (6). The sequence encoding the *cotB* promoter and part of the N-terminal region was amplified using the primers CotB5 (5′-GGCCATGGATCCACGGATTAGGCCGTTTGTCC-3′; the BamHI site is underlined) and CotB3 (5′-GGCCATAAGCTTGGATGATTGATCATCTGAAGATT-3′; the HindIII site is underlined). The amplified PCR product (831 bp) was inserted into the pDG1731 vector (28), which had previously been cleaved with BamHI and HindIII, giving rise to the pDGcotB vector. Next, the *slpA* and *invA* genes were amplified from the genomic DNA of *L. brevis* using the primers SlpAFw (5′-CAACGACTGCTAAGCTTATGAAGTCATACGCT-3′; the HindIII site is underlined) and SlpARv (5′-GGCCATGAATTCCGTTATCGTTGGTGGC-3′; the EcoRI site is underlined) and from the genomic DNA of *Y. pseudotuberculosis* using the primers InvAFw and InvA3 (5′-GGCCATGAATTCTATTGACAGCGCACAGAGCG-3′; the EcoRI site is underlined), respectively. The amplified PCR products (662 bp for *slpA* and 2.9 kb for *invA*) were digested with the restriction enzymes HindIII and EcoRI and cloned into pDGcotB, which had previously been digested, resulting in two integrative vectors, pLDV703 (SlpA) and pLDV704 (InvA) (Fig. 1a). All cloned sequences were confirmed by DNA sequencing. The integrative vectors were integrated at the nonessential *thrC* gene of the *B. subtilis* 1012 genome, resulting in strains LDV703* and LDV704* (Fig. 1a). The vaccine vector pLDV702 was constructed after cloning the *spaP1N* gene (1.4 kb), which encodes the $P1_{39-512}$ antigen from *S. mutans* strain UA159 (29), into the pHCMC03 vector under the control of P*gsiB*, which is active only during the vegetative growth stage (9, 30) (Fig. 1b). Finally, pLDV702 was introduced into *B. subtilis* strains 1012, LDV703*, and LDV704*, originating strains LDV702 (control), LDV703, and LDV704, respectively (Fig. 1a).

Preparation of *B. subtilis* spores. *B. subtilis* spores were recovered from cultures prepared in Foerster broth (31) supplemented with chloramphenicol (30 μg/ml) and incubated with aeration (200 rpm) at 37°C for 10 days. After cultivation, the spores were centrifuged and washed 3 times with sterile distilled water. The concentrations of the viable spores and vegetative cells were determined after plating serial dilutions of heat-treated samples (60 min at 65°C) on LB-chloramphenicol plates. Under the tested conditions, no contamination with vegetative cells was observed.

Extraction of spore coat proteins. The *B. subtilis* spore coat proteins were extracted as previously described (32). Briefly, spores were incubated in 50 mM dithiothreitol (DTT) and 1% sodium dodecyl sulfate (SDS) at 70°C for 30 min and then harvested at 8,000 × *g* for 10 min. Spore coat supernatant was used in enzyme-linked immunosorbent assays (ELISAs) and Western blotting assays, and both were performed with anti-SlpA or anti-InvA mouse polyclonal antibodies (kindly provided by Vasco Azevedo, Federal University of Minas Gerais, Brazil).

SDS-PAGE and Western blotting. Electrophoresis in denaturing gels was performed following a previously described protocol (33) and using a Mini-Protean II vertical electrophoresis unit (Bio-Rad). The Western blot assays were performed using previously described protocols (29). Reactive bands were detected with a chemiluminescence kit (SuperSignal; Pierce), as previously described (29).

Immunofluorescence of recombinant spores. Spores were fixed on glass slides using a previously described protocol (8). Briefly, the spores were suspended in GTE-lysozyme buffer (50 mM glucose, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mg/ml of lysozyme) and applied to glass slides previously treated with 0.01% poly-L-lysine (Sigma). After 5 min, excess liquid was removed. The slides were air dried, washed once with 1× phosphate-buffered saline (PBS), and blocked for 30 min with 1× PBS containing 2% bovine serum albumin (BSA). The spore samples were incubated for 1 h with specific anti-SlpA, anti-InvA polyclonal or preimmune sera, washed 3 times with PBS, and incubated with secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse IgG; Invitrogen, Carlsbad, CA) for 1 h. The stained cells were viewed using a Nikon TE300 inverted epifluorescence microscope (495-nm excitation, 525-nm emission).

Quantification of $P1_{39\text{-}512}$ by recombinant *B. subtilis* strains. $P1_{39\text{-}512}$ expression by the *B. subtilis* vaccine strains was monitored *in vitro* after induction by high-temperature incubation (42°C for 4 h), as previously described (30). Briefly, cells cultivated in LB broth at 37°C were shifted to 42°C at an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 and incubated for an additional 4 h. The cells were collected and suspended in lysis buffer (30% sucrose, 100 mM Tris HCl, pH 7.2, 800 μg/ml of lysozyme) for 30 min at 37°C, with SDS (final concentration, 1%) added to lyse the cells. The expressed $P1_{39\text{-}512}$ protein was measured by Western blotting and dot blotting using whole-cell lysates of different numbers of cells (10^2 to 10^7 CFU). Purified $P1_{39\text{-}512}$ was obtained as previously described (29) and used as a reference (3.2 to 250 ng of protein) for the quantification of the protein content in different *B. subtilis* strains. Reactive bands were analyzed using ImageJ software following the manufacturer's instructions.

Spore adhesion to Caco-2 cells. Adhesion of recombinant *B. subtilis* spores to gut epithelial cells was performed using the human Caco-2 colon carcinoma cell line (ATCC HTB37) according to a previously described procedure (34). The Caco-2 cells were cultured in RPMI medium supplemented with 2 mM L-glutathione and 20% fetal bovine serum. Spores (5×10^9 spores/well) were added to 24-well plates (Nunc, Thermo Scientific) containing Caco-2 cells at a concentration of 2×10^5 cells/well. The assay was performed with medium containing gentamicin (20 μg/ml) to inactivate germinated spores. Assays were performed after 2 h of incubation. The cells were lysed and heated at 65°C for 60 min, diluted in PBS, and, finally, plated on LB plates supplemented with chloramphenicol (30 μg/ml).

Transit of *B. subtilis* spores through the mouse intestinal tract. Groups of five BALB/c mice were inoculated with a single oral dose of 10^{11} spores of the tested *B. subtilis* strain. The mice were maintained in cages for 9 days. On each day, the fecal pellets were collected, weighed, and diluted in 5 ml of 1× PBS. The samples were centrifuged at 8,000 × *g* for 15 min (4°C), and the supernatants were serially diluted with PBS and plated before and after incubation for 60 min at 65°C on LB plates supplemented with spectinomycin (100 μg/ml) and chloramphenicol (30 μg/ml). The number of viable bacterial spores and vegetative cells per gram of feces was determined using the following formula: [(number of CFU/ml) × 5]/weight of feces collected.

Adhesion of *B. subtilis* spores to murine PPs. Groups of four BALB/c mice were inoculated with a single oral dose of 5×10^9 spores. After 1 or 6 days, the animals were sacrificed and the small intestine was aseptically removed. Fragments of the intestine were removed and washed gently with sterile saline, and the PPs were removed. The collected PPs were washed and treated with 0.2% Triton X-100 in saline. The whole-cell lysates were plated on LB plates containing spectinomycin (100 μg/ml) and chloramphenicol (30 μg/ml) before and after heat inactivation treatment for the determination of viable vegetative cells and spores. For histopathology analyses, PPs were aseptically removed, fixed with formalin (10%) for 24 h, placed in and processed in paraffin, and stained with hematoxylin-eosin.

Immunization regimens. All experiments involving animals were performed with prior approval from the Committee on the Ethical Use of Laboratory Animals of the Institute of Biomedical Sciences of São Paulo University (protocol no. 013) and in accordance with guidelines for the care and use of laboratory animals of the National Committee on the Ethics of Research (CONEP). For oral immunization, groups of five 6- to 8-week-old female BALB/c mice were inoculated with the spores of the different *B. subtilis* strains. The mice were immunized with 5×10^{10} spores diluted in bicarbonate solution (0.5 M) on days 1, 2, 3, 15, 16, 17, 29, 30, and 31. For sublingual immunizations, groups of BALB/c ($n = 5$) or Swiss ($n = 10$) mice (age, 6 to 8 weeks) were inoculated with the tested *B. subtilis* 1012, LDV702, or LDV704 strain on days 1, 15, and 30. The mice were anesthetized with ketamine (80 mg/kg of body weight) and xylazine hydrochloride (8 mg/kg of body weight) for sublingual immunization. Each dose contained 10^8 spores in a 10-μl volume, which was deposited under the tongue with the aid of a micropipette tip. For the nasal immunizations, the BALB/c mice ($n = 5$) received a dose of 10^8 spores in a 10-μl volume in the nares. Serum samples and saliva were collected 1 day before the first dose (preimmune samples), 1 day before each new set of doses, and 2 weeks after the last dose. Saliva was collected after the intraperitoneal (i.p.) injection of 0.5 ml of 0.2% pilocarpine to stimulate saliva flow. Phenylmethylsulfonyl fluoride (PMSF; 1 mM) was added to the samples to inhibit the action of proteases (35). All samples were stored at −80°C.

ELISA. The quantifications of the antigen-specific antibody levels in the serum and saliva samples collected from the immunized mice were performed by ELISA in 96-well MaxiSorp (Nunc) plates coated with the recombinant $P1_{39\text{-}512}$ protein according to previously described procedures (29). The absorbance values of the preimmune serum pools were used as blanks. Dilution curves were drawn for each sample, and the concentrations of antigen-specific antibodies were determined using standard curves prepared with known amounts of mouse IgG and IgA (Southern Biotech). All samples were assayed in duplicate.

Inhibition of *S. mutans* SAG-mediated adherence. Saliva-binding assays with the *S. mutans* NG8 strain were performed with purified salivary agglutinin (SAG), as described previously (36). The percentage of *S. mutans* adherence or adherence inhibition to immobilized SAG was determined as previously described (29). Briefly, purified SAG diluted in 1 volume of KPBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 6.5 mM Na_2HPO_4, pH 7.2) was adsorbed onto the wells (100 μl/well) of an Immobilon (Nunc) microtiter plate (4°C) for 16 h. The wells were washed once with TBSC (10 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.6), blocked with 1% BSA for 1 h at 25°C, and washed once again with TBSC. Aliquots (100 μl) of bacterial suspensions (prepared at an OD_{600} of 1 in

TBSC) were added to the wells, and the plates were incubated for 2 h at 37°C. The wells were washed twice with TBSC, and the adherent bacteria were fixed with 25% formaldehyde for 30 min at 25°C. The wells were washed twice with TBSC and stained with 0.5% crystal violet at 25°C for 1 min. Finally, the wells were washed, the dye was dissolved in 7% acetic acid, and the absorbance at 600 nm was measured. For adherence inhibition assays, the serum samples were mixed with the pretreated bacterial suspension and incubated for 30 min at 37°C, prior to the addition to the plate wells. The inhibition of *S. mutans* adhesion in the presence of immune serum was calculated as follows: $100 - [(A_{600}$ of NG8 cells incubated with the tested serum sample $\times$ 100)/A_{600} of NG8 cells not incubated with mouse serum]. Nonimmune serum and serum from mice immunized with the spores of *B. subtilis* strain 1012 were used as negative controls. All tested serum samples were collected after the complete immunization regimens and diluted to a final concentration of 2 μg/ml. All tests were performed in triplicate and independently repeated at least three times.

Statistical analyses. The results were analyzed using GraphPad Prism (version 5) software and are expressed as the mean ± standard deviation (SD). Statistically significant differences for the Caco-2 cell adhesion test were determined using Student's *t* test, whereas the other statistical analyses were performed with two-way analysis of variance followed by Bonferroni posttests.

RESULTS

Construction of recombinant *B. subtilis* spores capable of binding mammalian gut epithelial cells. The first step in the construction of the recombinant *B. subtilis* strains was the generation of recombinant *B. subtilis* spores expressing bacterial adhesins known to bind receptors found at gut epithelial cells at the spore surface. For that purpose, the N-terminal region of CotB, a surface-exposed spore protein (6), was genetically fused with two different adhesins with receptors at gut epithelial cells: the S-layer protein (SlpA) from *L. brevis*, which binds to fibronectin, and the invasin protein (InvA) from *Y. pseudotuberculosis*, which binds to integrin. These adhesins have been demonstrated to be functional when expressed in heterologous bacterial hosts (26, 27, 37, 38, 44). The integrative vectors, named pLDV703 (encoding the *cotB*::*slpA* fusion) and pLDV704 (encoding the *cotB*::*invA* fusion), were introduced by transformation into *B. subtilis* strain 1012, and stable recombinants were selected for the integration of the recombinant gene cassettes at the *thrC* locus, as previously described (28) (Fig. 1a). The spore surface display of SlpA (LDV703* strain) and InvA (LDV704* strain) was confirmed by Western blotting using spore coat protein extract preparations (Fig. 1c). Protein bands with apparent molecular masses of 54 kDa (corresponding to the molecular mass of the chimeric CotB-SlpA protein) and 137 kDa (corresponding to the molecular mass of the chimeric CotB-InvA protein) were detected in LDV703* and LDV704* spores, respectively (Fig. 1c). To confirm the spore surface localization of the bacterial adhesins, we performed immunofluorescence analyses with anti-SlpA and anti-InvA antibodies. A shown in Fig. 1d, we observed that the anti-SlpA and anti-InvA antibodies reacted specifically with the recombinant *B. subtilis* spores, confirming the display of the adhesins at the spore surface. No specific reaction was detected with preimmune sera. Similarly, no reaction was detected with the anti-SlpA and anti-InvA serum samples or with vegetative cells of the parenteral LDV702 strains (data not shown and Fig. 1d).

The second step in the construction of the recombinant *B. subtilis* strains involved the introduction of the gene encoding the target antigen, an N-terminal fragment of the *S. mutans* P1 protein ($P1_{39\text{-}512}$), under the control of a stress-inducible promoter (P*gsiB*) active only after spore germination (Fig. 1b) (9, 10). The pLDV702 plasmid carrying the *spaP1N*$_{39\text{-}512}$ gene was introduced into *B. subtilis* LDV703* and LDV704*, giving rise to the vaccine strains LDV703 and LDV704, respectively (Fig. 1a). The expression of $P1_{39\text{-}512}$ was monitored in whole-cell extracts of vegetative cells after a temperature shift (from 37°C to 42°C). All recombinant *B. subtilis* strains, including the control LDV702 strain, which did not express any surface-exposed spore adhesin, expressed the target antigen, as confirmed by Western blotting with P1-specific sera (Fig. 1e). The estimated amounts of $P1_{39\text{-}512}$ produced by the recombinant strains were 56.7 μg for LDV702 and LDV703 and 5.6 μg for LDV704 in a total amount of cells corresponding to 10^8 CFU (Fig. 1f).

TABLE 1 Elimination of spores and vegetative cells in mice orally dosed with spores from different *B. subtilis* strains

Strain[a]	Spores and cells[b]	Cells[b]	Spores[b]	% of initial inoculum[c]	Spore/cell ratio	Relative increase[d]
LDV702	9.5×10^4	1.0×10^4	8.5×10^4	1.9×10^{-5}	8.5	1
LDV703	1.4×10^8	4.5×10^7	9.8×10^7	2.8×10^{-2}	2.2	1.1×10^3
LDV704	1.4×10^8	2.5×10^7	1.2×10^8	2.8×10^{-2}	4.7	1.4×10^3

[a] Mice were orally dosed with 10^{11} spores.
[b] Numbers of spores or vegetative cells detected in the feces of mouse groups ($n = 5$) dosed with the different *B. subtilis* strains during the observation period (8 days).
[c] Percentage of cells and spores recovered in feces on the basis of the initial number of spores administered to the animals.
[d] Increase in the number of spores recovered from mice dosed with the different recombinant *B. subtilis* strains relative to the number of spores recovered from mice dosed with the parental LDV702 strain. The number of viable spores recovered from mice dosed with the LDV702 strain is represented as 1.

Expression of SlpA and InvA delays transit through the mouse intestinal tract and enhances the interaction of spores with Peyer's patches. To determine whether the SlpA and InvA adhesins expressed on the spore surface would retain biological activity and affect spore behavior, we incubated *B. subtilis* spores with human intestinal cells (of the Caco-2 human cell lineage). The results demonstrated that spores expressing SlpA and InvA interact *in vitro* with intestinal cells more efficiently than spores of the control LDV702 strain do (Fig. 2a). In addition, LDV703 and LDV704 spores persisted longer in the mouse intestinal tract, as demonstrated in BALB/c mice (Fig. 2b). LDV702 spores were detected in feces up to 72 h after ingestion, whereas spores of the recombinant LDV703 and LDV704 strains were excreted at high numbers ($>10^3$ spores/g feces) up to 8 days after administration (Fig. 2b). Taking into account the total number of spores and vegetative cells sampled from feces, mice orally dosed with spores of the LDV703 and LDV704 strains excreted 1,000-fold more bacteria and spores than mice dosed with spores of the LDV702 strain (Table 1). In addition, the proportion of spores in relation to the proportion of vegetative cells recovered in feces was higher in those animals ingesting spores of the LDV703 or LDV704 strain (spore/cell ratios, 2.2 and 4.7, respectively) than in mice dosed with spores of the control LDV702 strain (spore/cell ratio, 8.5) (Table 1). These results indicate that the expression of SlpA or InvA increased the transit of *B. subtilis* spores through the mouse intestinal tract, allowing the spores to experience more germination/sporulation cycles.

In the next step, we determined if the adhesive spores would in-

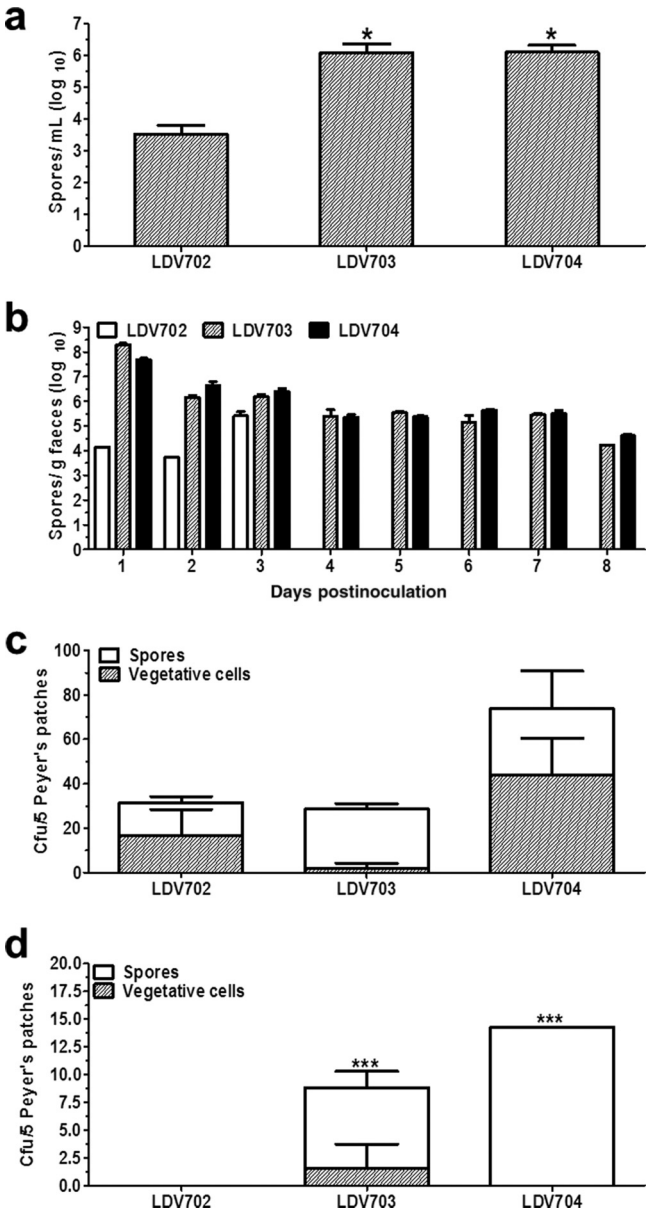

FIG 2 Functionality of SlpA and InvA expressed on the surface of *B. subtilis* spores. (a) Binding of *B. subtilis* spores to Caco-2 cells. Spores (5×10^9/well) were added to wells with cultured Caco-2 cells (2×10^5) and incubated for 2 h at 37°C. After extensive washings, the numbers of viable spores bound to the Caco-2 cells were determined by plating whole-cell homogenates. The results represent the average of three independent experiments, with each experiment performed in duplicate. (b) Intestinal transit of *B. subtilis* spores after oral administration to BALB/c mice. Mice ($n = 5$/group) were inoculated with a single oral dose (10^{11} spores/animal) of the spores suspended in bicarbonate solution. Fecal samples were collected periodically, diluted in saline, heated to inactivate vegetative cells, and plated on LB medium supplemented with antibiotics. (c, d) Interaction of *B. subtilis* spores with PPs. Spores (5×10^9) were given orally to BALB/c mice ($n = 4$). On days 1 (c) and 6 (d) postadministration, the animals were sacrificed. PPs were surgically removed, homogenized, and plated on LB supplemented with antibiotics for the determination of viable spores and vegetative cells. The assay was performed in duplicate and independently repeated at least three times. Values are expressed as means ± SDs, and the results obtained by statistical analyses represent the differences between the tested strains and the LDV702 strain (*, $P < 0.05$; ***, $P < 0.001$).

teract more efficiently with afferent sites of the murine GALT. For that purpose, we determined the number of *B. subtilis* spores detected in the PPs of mice orally dosed with spores. As indicated in Fig. 2c, 1 day after the spore administration, both the spores and cells of the three *B. subtilis* strains were detected in the PPs at similar numbers. Nonetheless, at 6 days after spore administration, only mice dosed with spores of the LDV703 or LDV704 strain contained viable spores in the PPs (Fig. 2d). No differences in the size or rough histological aspects were observed in the PPs of mice treated with spores of the LDV703 or LDV704 strain compared with mice treated with spores of the control LDV702 strain (data not shown). Altogether, these results indicate that the presence of bacterial adhesins at the spore coat enhanced the interaction of *B. subtilis* spores with the GALT afferent sites without inducing a measurable inflammatory response.

Oral administration of the adhesive spores improves antibody responses induced to a passenger vaccine antigen. To evaluate the impact of SlpA and InvA expression on the immunogenicity of an antigen carried by *B. subtilis* spores after mucosal administration, BALB/c mice were immunized via the oral route with recombinant spores capable of carrying the *S. mutans* $P1_{39\text{-}512}$ protein after spore germination. After the administration of one dose, only mice immunized with spores of the LDV703 or LDV704 strain developed measurable anti-$P1_{39\text{-}512}$ serum IgG responses (Fig. 3a). After a second dose, antigen-specific antibody responses were detected in mice immunized with spores of the three strains; however, animals immunized with adhesive spores developed statistically significantly higher responses than animals immunized with spores of the control strain (Fig. 3a). After the third dose, no significant differences in the anti-$P1_{39\text{-}512}$ serum IgG responses elicited in mice immunized with the spores of the three tested *B. subtilis* strains were detected. A noteworthy finding was that only mice orally dosed with spores of the LDV703 or LDV704 strain developed antigen-specific IgA responses in saliva after the third dose (Fig. 3b).

Because the P1 protein plays a pivotal role in the saliva-mediated agglutinin (SAG) adhesion of *S. mutans* to the tooth surface (17, 36), we determined if antibodies raised in mice immunized with the different *B. subtilis* vaccine strains would block the adhesion of *S. mutans* to human saliva immobilized on an abiotic surface. As demonstrated in Fig. 3c, serum samples collected from mice immunized with spores of the LDV703 or LDV704 strain blocked the adhesion of *S. mutans* to immobilized SAG by approximately 31% and 34%, respectively (Fig. 3c). In contrast, sera from mice immunized with LDV702 spores reduced the binding of *S. mutans* by only 18% (Fig. 3c). These results indicate that the expression of SlpA or InvA by *B. subtilis* spores increased the immunogenicity of $P1_{39\text{-}512}$ and improved the functionality of P1-specific antibodies, allowing a more efficient blockage of bacterial adhesion.

Enhanced immunogenicity of $P1_{39\text{-}512}$ after sublingual and nasal delivery of adhesive *B. subtilis* spores. We also tested whether the delivery/adjuvant effects of the *B. subtilis* spores could also be observed after the administration of spores via different mucosal routes. For this purpose, we evaluated immunization by the sublingual and nasal routes, previously shown to promote the induction of systemic and secreted antibody responses in murine models (39, 40). BALB/c mice were subjected to immunization regimens of three single doses, with each dose containing 10^8 spores of the LDV702 or LDV704 strain, delivered via the sublingual or nasal route. Two weeks after the last dose, mice immunized

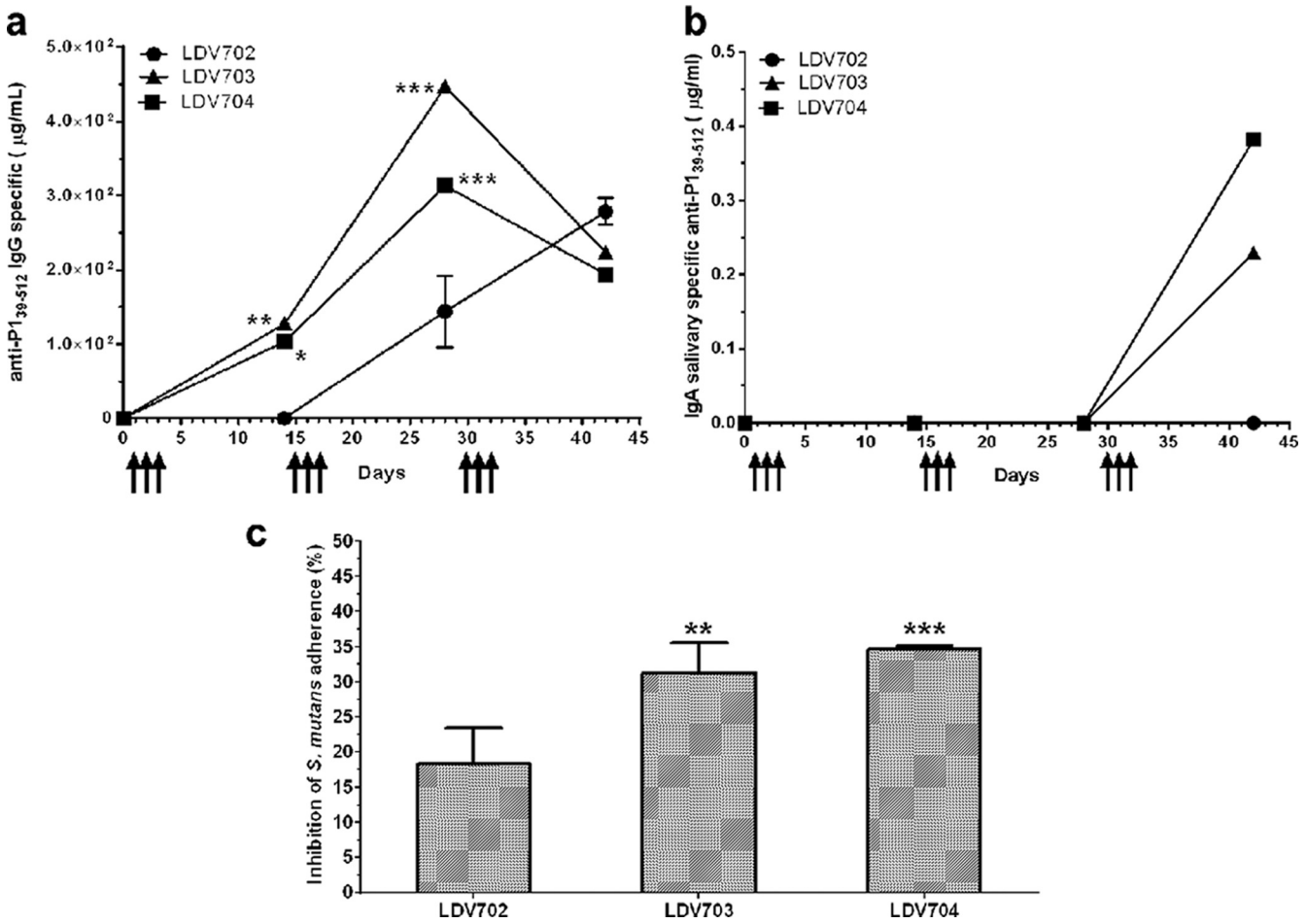

FIG 3 Antibody responses elicited in mice after oral administration of recombinant *B. subtilis* spores to inhibit binding of *S. mutans* cells to immobilized SAG. (a, b) Detection of P1-specific serum IgG (a) and salivary IgA (b) responses in mice immunized with recombinant *B. subtilis* spores via the oral route. Vaccine regimens were composed of 3 sets of 3 consecutive daily doses (5×10^{10} spores/dose) given at intervals of 2 weeks (arrows). P1$_{39-512}$-specific antibody responses in the serum and saliva samples were measured by ELISA. Values recorded in the sera of mice immunized with the nonrecombinant *B. subtilis* 1012 strain (negative control) were subtracted from the results obtained with the tested *B. subtilis* vaccine strains. (c) Inhibition of SAG-mediated adhesion of *S. mutans* by anti-P1$_{39-512}$-specific serum samples. The inhibition of adherence was determined using the following formula: $100 - [(A_{600}$ of NG8 cells incubated with the tested serum sample $\times 100)/A_{600}$ of NG8 cells not incubated with mouse serum]. All tested serum samples were obtained after the last dose and were used at the same concentration (2 μg/ml of P1-specific IgG). The results represent the averages of three independent experiments performed in triplicate. Statistically significant differences compared with the results obtained with the *B. subtilis* LDV702 strain are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

by the sublingual route developed higher anti-P1$_{39-512}$ serum IgG levels than mice orally immunized with 9 doses (10^{10} spores/dose) of spores of the same strains (Fig. 4a and b). Similar results were also observed in mice immunized with spores of the LDV702 and LDV704 strains via the nasal route. In addition, anti-P1$_{39-512}$ antibodies raised in immunized mice inhibited the binding of *S. mutans* to immobilized SAG, and this was particularly the case for those samples collected from mice immunized with LDV704 spores (Fig. 4d). To evaluate the immunogenicity of the recombinant spores among genetically heterogeneous animals, we repeated the immunizations with Swiss mice. For this purpose, mice were immunized by the sublingual route with recombinant spores of the LDV704 and LDV702 strains. For this step, we used the sublingual immunization route because previous results from our group and others have demonstrated that this administration route is safe and particularly efficient for inducing antigen-specific IgG responses (42, 43, 45). The results demonstrated that mice immunized with spores of the LDV704 and LDV702 strains developed high anti-P1$_{39-512}$ IgG responses (445 and 249 μg/ml, respectively) (Fig. 4c). In addition, anti-P1$_{39-512}$ antibodies raised in mice immunized with LDV704 spores inhibited the binding of *S. mutans* cells to immobilized SAG more efficiently (17% adherence inhibition) than antibodies raised in mice immunized with the LDV702 spores (3% adherence inhibition) (Fig. 4d). Altogether, these results demonstrate that the recombinant *B. subtilis* spores also displayed good performance as antigen delivery vectors in a genetically heterogeneous mouse population.

DISCUSSION

Despite the attractive features of bacterial spores as live vaccine vectors, the low immunogenicity of passenger antigens observed after oral administration to mammalian hosts represents a challenge to be faced before *B. subtilis* spores can be considered a viable alternative as a mucosal delivery system. Our results demonstrate that engineered *B. subtilis* spores can persist longer within the mammalian gastrointestinal tract, interact with GALT afferent

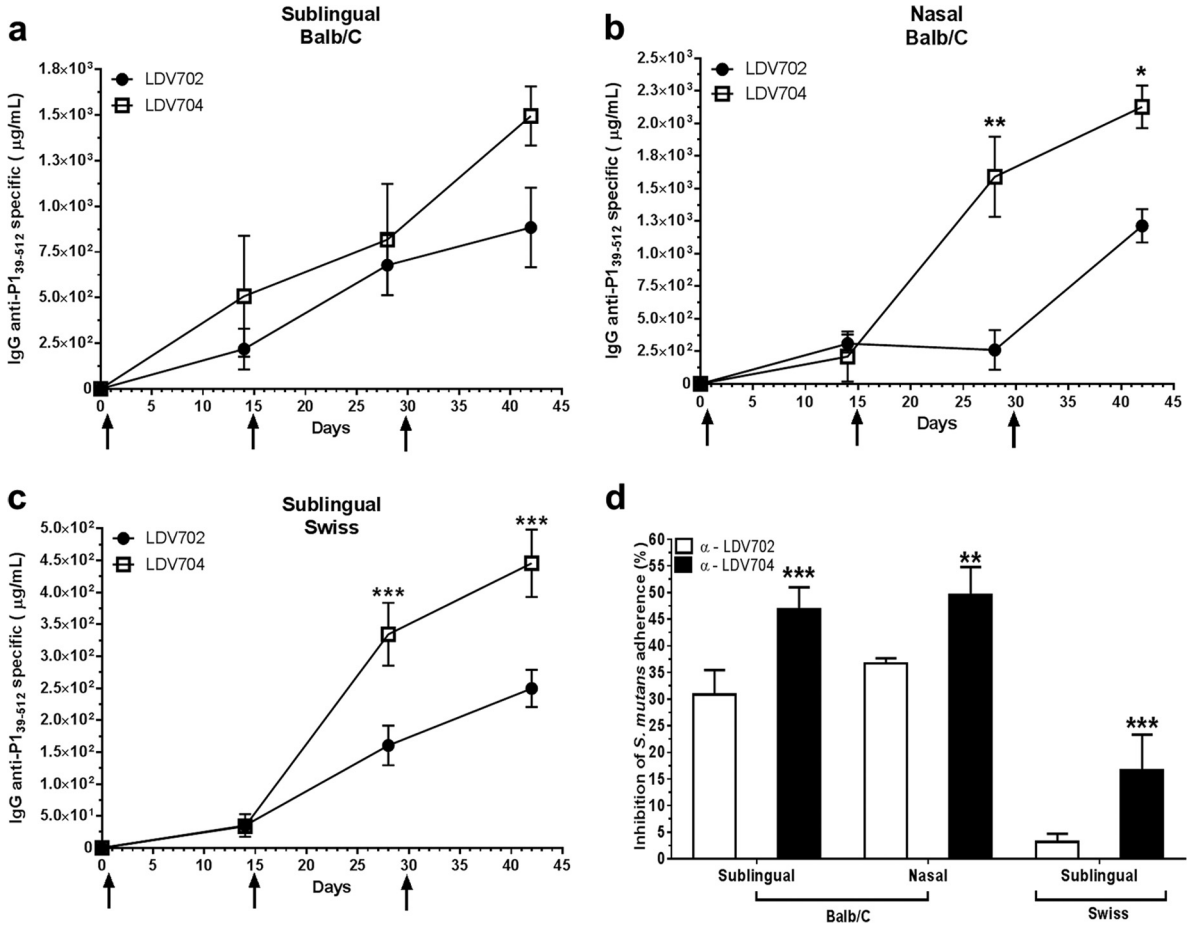

FIG 4 Antibody responses of mice immunized with *B. subtilis* spores via the sublingual or nasal route and neutralization of *S. mutans* SAG-mediated adherence to abiotic surfaces. (a, c) Systemic immune response elicited in BALB/c mice (a) and in Swiss mice (c) after immunization with *B. subtilis* spores by the sublingual route. (b) Anti-$P1_{39\text{-}512}$ IgG antibodies elicited in BALB/c mice immunized with *B. subtilis* spores by the nasal route. Mice were immunized with 3 single doses (10^8 spores/dose) of the *B. subtilis* LDV704 or LDV702 strain (arrows). The P1-specific IgG responses were measured by ELISA with pooled serum samples for the BALB/c mice ($n = 5$) and serum samples from individual animals for the Swiss mice ($n = 10$). The results obtained with *B. subtilis* 1012 were subtracted from the final values for the mice immunized with the vaccine strains. (d) Inhibition of SAG-mediated adhesion of *S. mutans* by anti-$P1_{39\text{-}512}$-specific antibodies. The inhibition of the adherence was determined using the same formula presented in the legend to Fig. 3. All tested serum samples were obtained after the last dose (pool) and were used at the same concentration (2 µg/ml of P1-specific IgG). Immunization groups are indicated in the graph (*x* axis). The results represent the averages of three independent experiments performed in triplicate. Statistically significant differences compared with the results obtained with the *B. subtilis* LDV702 strain are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

sites, and, more relevantly, improve the adjuvant effects of passenger antigens by the simple expression of bacterial adhesins at the spore surface. Expression of the binding region of the *L. brevis* S-layer protein (SlpA) and *Y. pseudotuberculosis* invasin (InvA) as gene fusions with a spore coat protein (CotB) enhanced serum and salivary antibody responses to $P1_{39\text{-}512}$ and increased the neutralizing effects of antibodies directed against *S. mutans* cells. Thus, the experimental evidence demonstrates that a single genetic manipulation step caused a drastic change in the behavior of *B. subtilis* spores and increased the perspectives for using spores as a platform for the mucosal delivery of heterologous antigens with diverse biotechnological applications.

Displaying adhesins capable of recognizing receptors expressed by gut epithelial cells not only increased the interaction of spores with GALT afferent sites but also enhanced the numbers of germination/sporulation cycles during transit through the murine gastrointestinal tract. The final numbers of spores and cells of the *B. subtilis* strains expressing surface-exposed adhesins were at least 3 orders of magnitude higher than those of the parental strain. Moreover, the spore/vegetative cell ratios measured with the recombinant strains were significantly lower than those detected with the parental strain (Table 1), which might result in higher antigen loads expressed *in vivo* (9, 10). This experimental evidence led us to propose a hypothetical model to explain the impact of SlpA expression and, particularly, InvA on the immunogenicity of antigens mucosally delivered by spores (Fig. 5). Adhesin-expressing spores would interact more efficiently with M cells, likely leading to the transcytosis of a large number of spores that are ultimately captured by intraepithelial antigen-presenting cells, such as macrophages and dendritic cells (Fig. 5). Once ingested by these phagocytic cells, as well as B cells, recombinant spores would germinate in phagosomes, where the activation of the *gsiB* promoter would drive antigen synthesis either to follow conventional processing and presentation pathways, leading to the activation of effector T and B cells, or as an antigen source for cross-presentation to intraepithelial B cells. As a final consequence, the local and

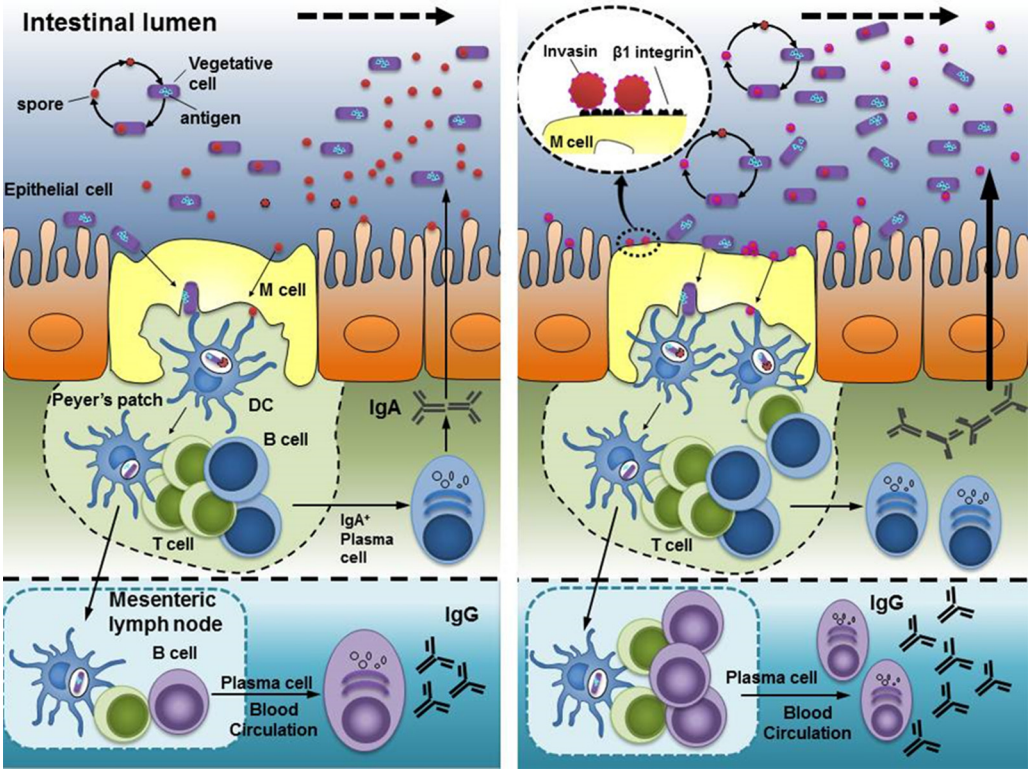

FIG 5 Differential behavior of recombinant *B. subtilis* spores displaying SlpA or InvA adhesins and the induction of immune responses to a passenger antigen. (Left) Gastrointestinal transit and interaction with GALT afferent sites of nonrecombinant *B. subtilis* spores. DC, dendritic cell. (Right) Gastrointestinal transit and interaction with GALT afferent sites of *B. subtilis* spores expressing SlpA or InvA. Recombinant *B. subtilis* spores are targeted to the gut epithelium and GALT afferent sites, leading to enhanced immune responses and antigen-specific antibodies with higher epitope specificities.

systemic production of antigen-specific antibody responses would be enhanced, as demonstrated by the augmented production of anti-$P1_{39\text{-}512}$ salivary IgA and serum IgG. Interestingly, previous attempts to employ a natural *B. subtilis* strain capable of persisting longer in the gut gastrointestinal tract of mice did not enhance the immunogenicity of a target antigen fused at the spore coat (43). Therefore, it is possible to infer that specific spore targeting to GALT afferent sites, mediated by surface-exposed adhesins, and the expression of the vaccine antigen after spore germination represent key features involved in the higher immunogenicity of the recombinant *B. subtilis* spores.

To further evaluate the versatility of this spore-based mucosal antigen delivery method, we tested the performance of the adhesive spores using two alternative mucosal administration routes. Recombinant spores administered via the sublingual and nasal routes resulted in a dramatic enhancement in the immunogenicity of the passenger antigen, with high serum antibody responses being achieved even after a single vaccine dose containing 10^8 spores (a 100-fold smaller dose in comparison with that administered by the oral route). Such a performance regarding the induction of antigen-specific antibody responses is similar to that achieved with mucosa-delivered attenuated bacterial species, such as *Salmonella*. Indeed, the advantageous use of the sublingual route for the administration of *B. subtilis* spores has been emphasized previously (41, 42). Our results also demonstrate that an enhanced immune response to a passenger antigen can also be achieved in a genetically heterogeneous mouse strain, further supporting the use of recombinant *B. subtilis* as a new platform for the delivery of antigens via mucosal routes.

Currently, two experimental approaches have been developed for the generation of *B. subtilis* spores as live vaccine vectors: one is based on the expression of the target antigen as a fusion protein at the spore coat (6–8, 13, 43), whereas the second involves expression of the antigen after spore germination (9–11). The present results represent the first demonstration of a combination of two antigen expression strategies: one active during spore formation, leading to the surface display of recombinant adhesins at the spore surface, and a second pathway leading to the intracellular expression of the target antigen shortly after spore germination. The combination of the two heterologous protein expression systems significantly enhanced the immunogenicity of antigens delivered by *B. subtilis* spores following administration via mucosal routes. The discovery that the immunological performance of *B. subtilis* spores as antigen delivery vectors can be significantly improved by expressing bacterial adhesins, particularly InvA, on the spore surface therefore represents the basis of a second-generation spore-based antigen delivery system endowed with enhanced adjuvant effects and compatible with administration via different mucosal routes. In addition, the *S. mutans* neutralization effects achieved with the presently reported antigen expression strategy open new and promising avenues for the development of anticaries vaccines.

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REFERENCES

1. **Unnikrishnan M, Rappuoli R, Serruto D.** 2012. Recombinant bacterial vaccines. Curr. Opin. Immunol. **24:**337–342. http://dx.doi.org/10.1016/j.coi.2012.03.013.
2. **Medina M, Vintiñi E, Villena J, Raya R, Alvarez S.** 2010. *Lactococcus lactis* as an adjuvant and delivery vehicle of antigens against pneumococcal respiratory infections. Bioeng. Bugs **1:**313–325. http://dx.doi.org/10.4161/bbug.1.5.12086.
3. **Drabner B, Guzmán CA.** 2001. Elicitation of predictable immune responses by using live bacterial vectors. Biomol. Eng. **17:**75–82. http://dx.doi.org/10.1016/S1389-0344(00)00072-1.
4. **Hoa TT, Duc LH, Isticato R, Baccigalupi L, Ricca E, Van PH, Cutting SM.** 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ. Microbiol. **67:**3819–3823. http://dx.doi.org/10.1128/AEM.67.9.3819-3823.2001.
5. **Ferreira LC, Ferreira RC, Schumann W.** 2005. *Bacillus subtilis* as a tool for vaccine development: from antigen factories to delivery vectors. An. Acad. Bras. Cienc. **77:**113–124. http://dx.doi.org/10.1590/S0001-37652005000100009.
6. **Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, Oggioni MR, De Felice M, Pozzi G, Ricca E.** 2001. Surface display of recombinant proteins on *Bacillus subtilis* spores. J. Bacteriol. **183:**6294–6301. http://dx.doi.org/10.1128/JB.183.21.6294-6301.2001.
7. **Mauriello EM, Duc le H, Isticato R, Cangiano G, Hong HA, De Felice M, Ricca E, Cutting SM.** 2004. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. Vaccine **22:**1177–1187. http://dx.doi.org/10.1016/j.vaccine.2003.09.031.
8. **Duc le H, Hong HA, Fairweather N, Ricca E, Cutting SM.** 2003. Bacterial spores as vaccines vehicles. Infect. Immun. **71:**2810–2818. http://dx.doi.org/10.1128/IAI.71.5.2810-2818.2003.
9. **Paccez JD, Luiz WB, Sbrogio-Almeida ME, Ferreira RC, Schumann W, Ferreira LC.** 2006. Stable episomal expression system under control of a stress inducible promoter enhances the immunogenicity of *Bacillus subtilis* as a vector for antigen delivery. Vaccine **24:**2935–2943. http://dx.doi.org/10.1016/j.vaccine.2005.12.013.
10. **Paccez JD, Nguyen HD, Luiz WB, Ferreira RC, Sbrogio-Almeida ME, Schuman W, Ferreira LC.** 2007. Evaluation of different promoter sequences and antigen sorting signals on the immunogenicity of *Bacillus subtilis* vaccine vehicles. Vaccine **25:**4671–4680. http://dx.doi.org/10.1016/j.vaccine.2007.04.021.
11. **Luiz WB, Cavalcante RC, Paccez JD, Souza RD, Sbrogio-Almeida ME, Ferreira RC, Ferreira LC.** 2008. Boosting systemic and secreted antibody responses in mice orally immunized with recombinant *Bacillus subtilis* strains following parenteral priming with a DNA vaccine encoding the enterotoxigenic *Escherichia coli* (ETEC) CFA/I fimbriae B subunit. Vaccine **26:**3998–4005. http://dx.doi.org/10.1016/j.vaccine.2008.05.030.
12. **Ceragioli M, Cangiano G, Esin S, Ghelardi E, Ricca E, Senesi S.** 2009. Phagocytosis, germination and killing of *Bacillus subtilis* spores presenting heterologous antigens in human macrophages. Microbiology **155:**338–346. http://dx.doi.org/10.1099/mic.0.022939-0.
13. **Duc le H, Hong HA, Uyen NQ, Cutting SM.** 2004. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine **22:**1873–1885. http://dx.doi.org/10.1016/j.vaccine.2003.11.021.
14. **Yasui T, Yoda K, Kamiya T.** 1995. Analysis of S-layer proteins of *Lactobacillus brevis*. FEMS Microbiol. Lett. **133:**181–186. http://dx.doi.org/10.1111/j.1574-6968.1995.tb07881.x.
15. **Young VB, Falkow S, Schoolnik GK.** 1992. The invasin protein of *Yersinia enterocolitica*: internalization of invasin-bearing bacteria by eukaryotic cells is associated with reorganization of the cytoskeleton. J. Cell Biol. **116:**197–207. http://dx.doi.org/10.1083/jcb.116.1.197.
16. **Okahashi N, Sasakawa C, Yoshikawa M, Hamada S, Koga T.** 1989. Molecular characterization of a surface protein antigen gene from serotype c *Streptococcus mutans*, implicated in dental caries. Mol. Microbiol. **3:**673–678. http://dx.doi.org/10.1111/j.1365-2958.1989.tb00215.x.
17. **Crowley PJ, Brady LJ, Michalek SM, Bleiweis AS.** 1999. Virulence of a spaP mutant of *Streptococcus mutans* in a gnotobiotic rat model. Infect. Immun. **67:**1201–1206.
18. **Zhang S, Green NM, Sitkiewicz I, Lefebvre RB, Musser JM.** 2006. Identification and characterization of an antigen I/II family protein produced by group A *Streptococcus*. Infect. Immun. **74:**4200–4213. http://dx.doi.org/10.1128/IAI.00493-06.
19. **Brady LJ, Maddocks SE, Larson MR, Forsgren N, Persson K, Deivanayagam CC, Jenkinson HF.** 2010. The changing faces of *Streptococcus* antigen I/II polypeptide family adhesins. Mol. Microbiol. **77:**276–286. http://dx.doi.org/10.1111/j.1365-2958.2010.07212.x.
20. **Wehrl W, Niederweis M, Schumann W.** 2000. The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation. J. Bacteriol. **182:**3870–3873. http://dx.doi.org/10.1128/JB.182.13.3870-3873.2000.
21. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. **Cutting SM, Oke V, Driks A, Losick R, Lu S, Kroos L.** 1990. A forespore checkpoint for mother cell gene expression during development in *B. subtilis*. Cell **62:**239–250. http://dx.doi.org/10.1016/0092-8674(90)90362-I.
23. **Dersch P, Isberg RR.** 1999. A region of the *Yersinia pseudotuberculosis* invasin protein enhances integrin-mediated uptake into mammalian cells and promotes self-association. EMBO J. **18:**1199–1213. http://dx.doi.org/10.1093/emboj/18.5.1199.
24. **Leong JM, Fournier RS, Isberg RR.** 1990. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. EMBO J. **9:**1979–1989.
25. **Clark MA, Hirst BH, Jepson MA.** 1998. M-cell surface B-integrin expression and invasin-mediated targeting of *Yersinia pseudotuberculosis* to mouse Peyer's patch M cells. Infect. Immun. **66:**1237–1243.
26. **Hynönen U, Westerlund-Wikström B, Palva A, Korhonen TK.** 2002. Identification by flagellum display of an epithelial cell- and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. J. Bacteriol. **184:**3360–3367. http://dx.doi.org/10.1128/JB.184.12.3360-3367.2002.
27. **Avall-Jääskeläinen S, Lindholm A, Palva A.** 2003. Surface display of the receptor-binding region of the *Lactobacillus brevis* S-layer protein in *Lactococcus lactis* provides nonadhesive lactococci with the ability to adhere to intestinal epithelial cells. Appl. Environ. Microbiol. **69:**2230–2236. http://dx.doi.org/10.1128/AEM.69.4.2230-2236.2003.
28. **Guérout-Fleury AM, Frandsen N, Stragier P.** 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57–61. http://dx.doi.org/10.1016/S0378-1119(96)00404-0.
29. **Tavares MB, Silva BM, Cavalcante RC, Souza RD, Luiz WB, Paccez JD, Crowley PJ, Brady LJ, Ferreira LC, Ferreira RC.** 2010. Induction of neutralizing antibodies in mice immunized with an amino-terminal polypeptide of *Streptococcus mutans* P1 protein produced by a recombinant *Bacillus subtilis* strain. FEMS Immunol. Med. Microbiol. **59:**131–142. http://dx.doi.org/10.1111/j.1574-695X.2010.00669.x.
30. **Phan TT, Nguyen HD, Schumann W.** 2006. Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in *Bacillus subtilis*. Protein Expr. Purif. **46:**189–195. http://dx.doi.org/10.1016/j.pep.2005.07.005.
31. **Foerster HF, Foster JW.** 1966. Endotrophic calcium, strontium, and barium spores of *Bacillus megaterium* and *Bacillus cereus*. J. Bacteriol. **91:**1333–1345.
32. **Nicholson WL, Setlow P.** 1990. Sporulation, germination and outgrowth, p 391–450. *In*Harwood CR, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons, Sussex, England.
33. **Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685. http://dx.doi.org/10.1038/227680a0.
34. **Dramsi S, Biswas I, Maguin E, Braun L, Mastroeni P, Cossart P.** 1995. Entry of *Listeria monocytogenes* into hepatocytes requires expression of InIB, a surface protein of the internalin multigene family. Mol. Microbiol. **16:**251–261. http://dx.doi.org/10.1111/j.1365-2958.1995.tb02297.x.
35. **Jia R, Guo JH, Fan MW, Bian Z, Chen Z, Peng B, Fan B.** 2004. Mucosal immunization against dental caries with plasmid DNA encoding pac gene of *Streptococcus mutans* in rats. Vaccine **22:**2511–2516. http://dx.doi.org/10.1016/j.vaccine.2004.01.025.
36. **Brady LJ, Piacentini DA, Crowley PJ, Oyston PC, Bleiweis AS.** 1992. Differentiation of salivary agglutinin-mediated adherence and aggregation of mutans streptococci by use of monoclonal antibodies against the major surface adhesin P1. Infect. Immun. **60:**1008–1017.
37. **Dramsi S, Kocks C, Forestier C, Cossart P.** 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator prfA. Mol. Microbiol. **9:**931–941. http://dx.doi.org/10.1111/j.1365-2958.1993.tb01223.x.
38. **Avall-Jääskeläinen S, Kylä-Nikkilä K, Kahala M, Miikkulainen-Lahti T, Palva A.** 2002. Surface display of foreign epitopes on the *Lactobacillus*

brevis S-layer. Appl. Environ. Microbiol. **68:**5943–5951. http://dx.doi.org/10.1128/AEM.68.12.5943-5951.2002.

39. **Hervouet C, Luci C, Çuburu N, Cremel M, Bekri S, Vimeux L, Marañon C, Czerkinsky C, Hosmalin A, Anjuère F.** 2010. Sublingual immunization with an HIV subunit vaccine induces antibodies and cytotoxic T cells in mouse female genital tract. Vaccine **28:**5582–5590. http://dx.doi.org/10.1016/j.vaccine.2010.06.033.
40. **Czerkinsky C, Çuburu N, Kweon MN, Anjuere F, Holmgren J.** 2011. Sublingual vaccination. Hum. Vaccin. **7:**110–114. http://dx.doi.org/10.4161/hv.7.1.13739.
41. **Amuguni JH, Lee S, Kerstein KO, Brown DW, Belitsky BR, Herrmann JE, Keusch GT, Sonenshein AL, Tzipori S.** 2011. Sublingually administered Bacillus subtilis cells expressing tetanus toxin C fragment induce protective systemic and mucosal antibodies against tetanus toxin in mice. Vaccine **29:**4778–4784. http://dx.doi.org/10.1016/j.vaccine.2011.04.083.
42. **Amuguni JH, Lee S, Kerstein K, Brown D, Belitsky B, Herrmann J, Keusch G, Sonenshein A, Tzipori S.** 2012. Sublingual immunization with an engineered *Bacillus subtilis* strain expressing tetanus toxin fragment C induces systemic and mucosal immune responses in piglets. Microbes Infect. **14:**447–456. http://dx.doi.org/10.1016/j.micinf.2011.12.001.
43. **Uyen NQ, Hong HA, Cutting SM.** 2007. Enhanced immunisation and expression strategies using bacterial spores as heat-stable vaccine delivery. Vaccine **25:**356–365. http://dx.doi.org/10.1016/j.vaccine.2006.07.025.
44. **Critchley-Thorne RJ, Stagg AJ, Vassaux G.** 2006. Recombinant *Escherichia coli* expressing invasin targets the Peyer's patches: the basis for a bacterial formulation for oral vaccination. Mol. Ther. **14:**183–191. http://dx.doi.org/10.1016/j.ymthe.2006.01.011.
45. **Lee S, Belitsky BR, Brown DW, Brinker JP, Kerstein KO, Herrmann JE, Keusch GT, Sonenshein AL, Tzipori S.** 2010. Efficacy, heat stability and safety of intranasally administered *Bacillus subtilis* spore or vegetative cell vaccines expressing tetanus toxin fragment C. Vaccine **28:**6658–6665. http://dx.doi.org/10.1016/j.vaccine.2010.08.016.