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Stable episomal expression system under control of a stress inducible promoter enhances the immunogenicity of *Bacillus subtilis* as a vector for antigen delivery

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Abstract

Bacillus subtilis has been successfully engineered to express heterologous antigens genetically fused to surface-exposed spore coat proteins as a vaccine vehicle endowed with remarkable heat resistance and probiotic effects for both humans and animals. Nonetheless, the immunogenicity of passenger antigens expressed by *B. subtilis* spores is low particularly following oral delivery. In this work, we describe a new episomal expression system promoting enhanced immunogenicity of heterologous antigens carried by *B. subtilis* strains, either in the form of spores or vegetative cells, following oral or parenteral delivery to mice. Based on a bi-directional replicating multicopy plasmid, the gene encoding the B subunit of the heat-labile toxin (LTB), produced by enterotoxigenic *Escherichia coli* (ETEC) strains, was cloned under the control of the *B. subtilis* glucose starvation inducible (*gsiB*) gene promoter, active in vegetative cells submitted to heat and other stress conditions. The recombinant plasmid proved to be structurally and segregationally stable in both cells and spores under in vitro and in vivo conditions. Moreover, BALB/c mice orally immunized with *B. subtilis* cells or spores elicited enhanced anti-LTB systemic (serum IgG) and secreted (fecal IgA) antibody responses, thus, suggesting that antigen expression occurred during in vivo transit. These results indicate that the new episomal expression system may improve the performance of *B. subtilis* as a live orally-delivered vaccine carrier.

Keywords: *B. subtilis* spores; *B. subtilis* vaccine vectors; *gsiB*; Oral vaccines; LT; ETEC

1. Introduction

Among various live vaccine bacterial vectors so far tested, recombinant *Bacillus subtilis* strains are one of the most recent additions [1,2]. In contrast to attenuated Gram-negative species, such as *Salmonella* and *Shigella*, *B. subtilis* has been granted the GRAS status, and the available knowledge on genetics and physiology of this species finds a parallel only in *Escherichia coli* K12. Moreover, *B. subtilis* has the distinguishing characteristic of producing spores, the most resilient life form found on the planet and with a long standing history of probiotic use in humans and animals [3]. Recent reports describing the use of engineered *B. subtilis* spores expressing heterologous antigens genetically fused to surface exposed spore coat proteins raised considerable interest for those aiming the development of orally delivered vaccines. Nonetheless, the need of repeated immunizations for proper activation of antibody responses indicates that the immunogenicity of *B. subtilis*, either in the form of spores or vegetative cells, is low [1,2,4].

The low immunogenic efficacy of *B. subtilis* can be mainly attributed to at least two main factors. First, *B. subtilis* is not

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a typical inhabitant of the mammalian intestinal microbiota. Orally ingested *B. subtilis* cells are eliminated within 48 h after ingestion while spores are completely shed in approximately 1 week [2,5–7]. Thus, productive interaction of bacteria or spores with gut-associated lymphoid tissue (GALT) afferent sites is brief, requiring multiple highly concentrated doses, potentially leading to activation of immune tolerance. A second factor contributing to the reduced immunogenicity of *B. subtilis* as a vaccine vector is the amount of expressed antigen presented to the mammalian immune system. Multicopy plasmid-based expression vectors for Gram-positive bacteria are normally unstable reflecting both segregational loss of the plasmid or structural unstable expression caused by intramolecular recombination events [8]. Most convenient *B. subtilis* expression plasmids have been derived from *Staphylococcus aureus* plasmids, which replicates via single-stranded DNA intermediates capable of suffering rearrangements after cloning of heterologous sequences [8]. Integrative vectors carrying an expression cassette sandwiched between the two halves of a non-essential gene, such as *amyE,* have been used as an approach to avoid the genetic instability of episomal expression systems [9]. Although integration of recombinant gene cassettes into the *B. subtilis* chromosome confers enhanced stability to the gene expression the reduced copy number decreases the antigen load carried by recombinant cells or spores.

Here we report the development of an alternative stable episomal expression system for *B. subtilis* capable of increasing the immunogenicity of orally delivered vaccine strains either as vegetative cells or spores. Based on a recently described *E. coli–B. subtilis* shuttle vector capable of replicating in *B. subtilis* via a double-stranded DNA intermediate [10], we constructed an expression vector under the control of a stress-inducible promoter derived from the *B. subtilis* glucose starvation inducible (*gsiB*) gene [11,12]. The *gsiB* promoter is recognized by an alternative sigma factor (σB), activated when cells are submitted to different environmental stresses such as heat, low pH, glucose starvation and anaerobiosis [11,12]. The amount of antigen produced by plasmid transformed *B. subtilis* cells was enhanced and stabilized both under in vivo and in vitro conditions. Moreover, although not active during the sporulation phase, the *gsiB* promoter proved to be induced during the transit through the mammalian host. As a model antigen we have chosen the heat-labile toxin B subunit (LTB) produced by some enterotoxigenic *E. coli* (ETEC) strains, an important etiological agent of infantile diarrhea and the single most relevant pathogen associated with traveler's diarrhea [13]. Indeed, ETEC LTB has a rather extensive record of systemic and secreted immunogenicity when delivered by *B. subtilis* and other Gram-positive bacterial species [4,14]. The results described herein demonstrate that the new expression system enhances the immunogenicity of LTB expressed by *B. subtilis* either in the form of spores or vegetative cells and may contribute to the development of more effective live vaccine vectors.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *B. subtilis* WW02 strain (*leuA8 metB5 trpC2 hsrdRM1 amyE::neo*) was used for all immunization experiments [15]. The *B. subtilis* LDV1 and LDV2 and LDV3 strains were obtained after transformation with the pLDV1, pLDV2 and pREP9 [16] expression vectors, respectively. All cloning steps were carried out with the *E. coli* DH5α strain. Bacterial strains were routinely grown in luria-broth (LB) and plates were prepared with added neomycin (25 μg/ml) and/or chloramphenicol (5 μg/ml), for *B. subtilis*, or ampicillin (100 μg/ml) for *E. coli*. Sporulation of the *B. subtilis* strains was induced in difco-sporulation media (DSM) using the exhaustion method as previously described [17]. *E. coli* competent cells were prepared with the $CaCl_2$-mediated transformation protocol, while *B. subtilis* competent cells were obtained by the two-step transformation method [18,19].

2.2. Plasmid construction

The *gsiB* gene upstream region, encompassing the promoter and ribosome-binding site, was amplified with primers ON3 (5′ GGC CAT GGA TCC CTA TCG AGA CAC GTT TGG CTG 3′) and ON4 (5′GGC CAT GAG CTC TTC CTC CTT TAA TTG GTG TTG GT 3′, restriction sites underlined) and cloned into *Sac*I-*Bam*HI double-digested pMTLBS72 [10]. After restriction analysis, one clone containing the insert was chosen and the recombinant plasmid, named pLDV1, isolated for a final cloning step with an amplified fragment containing the *elTB* gene derived from ETEC H10407 strain [20]. Amplification of the *eltB* was carried out with primers ELTBFw (5′ TCT ATG TAG ATC TAT GGC TCC TCA GTC TAT TAC AGA 3′) and ELTB2Rv (5′ TTT TAA TTC TAG ATT AGT TTT CCA TAC TGA TTG CCG C 3′). Once digested with *Bam*HI and *Xba*I, the amplified fragment was forced cloned into *Bam*HI/*Xba*I cleaved pLDV1. One recombinant plasmid was selected, subjected to restriction analysis and nucleotide sequencing and named pLDV2 (Fig. 1). Both restriction analysis and necleotide sequencing confirmed the correct cloning of the *eltB* gene.

2.3. In vitro expression of LTB by B. subtilis pLDV2

Wild-type and recombinant *B. subtilis* strains were grown in LB in Erlenmeyer flasks aerated in an orbital shaker set at 200 rpm at 28 °C overnight. New cultures were prepared after diluting cells (1:100) in fresh medium kept at 28 °C under aeration until an OD_{600nm} of 0.6–0.8 was reached. Heat-shocked cells were submitted to a temperature shift to 45 °C for 2 h. Whole cell extracts were prepared after incubation of cells, corresponding to an OD_{600nm} of 2.2, in lysis buffer (15% sucrose, 250 mM Tris–HCl pH 7.5, lysozyme 800 μg/ml) for 5 min followed by addition of 10 μl of 10% SDS and incubation at 37 °C for 15 min. SDS–PAGE was

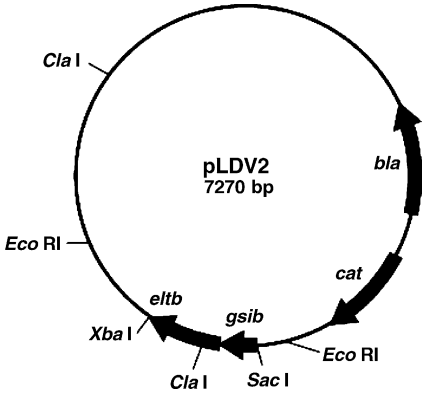

Fig. 1. Schematic representation of the *B. subtilis* expression vector pLDV2. Positions of unique cloning sites, the *gsiB* promoter and genes encoding resistance to ampicillin (*bla*), chloramphenicol (*cat*) as well as the *eltB* gene structural are indicated.

performed following standard procedures in a Mini Protean II vertical electrophoresis unit (Bio-Rad). Samples were boiled with an equal amount of sample buffer (0.625 M Tris pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% mercaptoethanol in distilled water) for 5 min and applied to 15% (w/v) polyacrylamide gels. Gels were run at 120 V and the sorted proteins transferred to nitrocellulose sheets (0.45 μm pore size, Sigma) at 200 mA for 1 h based on previously described conditions [21]. After overnight blocking with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4 °C, the nitrocellulose sheets were incubated at room temperature for 1 h with anti-LT specific serum followed, after three washing steps, by incubation with 1:3,000 PBS-diluted rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma). Reactive bands were developed with a chemoluminescence kit (Super Signal, Pierce), as specified by the manufacturer, and exposed to Kodak X-Omat films for 1–5 min.

2.4. Preparation of spores

Sporulation of *B. subtilis* was carried out in DSM using the exhaustion method as described previously [17]. Cultures were harvested 24 h after sporulation onset, subjected to lysozyme treatment to break any residual cells, followed by successive washes in 1 M NaCl and 1 M KCl and then washed twice in water. PMSF (10 mM) was included in washes to inhibit proteolysis. Finally, the spores were suspended in water and treated at 68 °C for 1 h to inactivate any residual cells. Viable spores were titrated for determination of the number of CFU/ml and then transferred to −20 °C until use.

2.5. Determination of plasmid stability in B. subtilis under in vitro and in vivo conditions

Segregational stabilities of plasmids pLDV2 and pREP9 were evaluated under in vitro with cells cultivated in LB medium without antibiotics at 37 °C under aeration. New cultures were prepared by dilution of overnight grown cells in approximately 1000 CFU/ml. The procedure was repeated during a period of 7 days corresponding to approximately 225 generations. Plasmid-containing cells were detected after replica-plating neomycin-resistant colonies (the *neo* gene is located within the chromosome) on agar plates containing chloramphenicol (5 μg/ml), the antibiotic resistance marker encoded by pLDV2 and pREP9. All neomycin-resistant and chloramphenicol-sensitive colonies were considered cured of the tested plasmid, and as much as 200 colonies were tested per interval. Sets of 10 chloramphenicol resistant colonies were also tested for LTB expression after incubation at 45 °C and Western blot analysis. Plasmid stability under in vivo conditions was measured in groups of five female mice inoculated with a single per oral (p.o.) dose of 10^{10} CFU of *B. subtilis* cells or spores. Mice were kept in gridded floor cages to prevent coprophagia and fecal pellets were harvested at daily intervals for periods up to 72 h after the inoculation. Pellets were homogenized (1:10) in PBS, submitted to serial dilutions in PBS, plated on DSM agar plates containing neomycin and, then, replica-plated in neomycin/chloramphenicol containing plates. In mice dosed with *B. subtilis* spores, fecal suspensions were incubated at 65 °C for 1 h to eliminate vegetative cells. The number of tested colonies varied from 20 to 1500 according to the tested time points. Sets of 5–10 chloramphenicol resistant colonies were also submitted to Western blot experiments to evaluate LTB expression.

2.6. Immunization regimens

C57BL/6 female mice were supplied by the Isogenic Mouse Breeding Facility of the Department of Immunology, Biomedical Sciences Institute (ICB), University of São Paulo (USP). All animal handling was in accordance with the principles of the Brazilian code for the use of laboratory animals. Groups of five 8 weeks old female mice were inoculated per oral or intraperitoneally (i.p.) with vegetative cells or spores of the *B. subtilis* strains transformed with pLDV1 or pLDV2. p.o. immunizations were carried out with 0.5 ml aliquots of bacterial suspensions containing approximately 3×10^{10} CFU of vegetative cells or 1.5×10^{10} spores using a stainless-steel round tip gavage cannule. Mice submitted to the p.o. immunizations received 0.5 ml of a 0.1 M sodium bicarbonate solution 30 min before the administration of the bacterial or spore vehicles. The parenteral immunizations were performed with 2×10^9 CFU of vegetative cells or 10^9 spores suspended in PBS in a final volume of 0.2 ml. The immunization regimens for both cells and spores were based on previously reported attempts to use *B. subtilis* as vaccine vehicles [2,4]. Mice immunized via the p.o. route received one or three doses consisting of three sets of three consecutive doses on days. The single dose immunization regimen consisted of three daily doses given on Days 1–3, while in the three dose regimen mice receive doses on Days 1–3, 14–16, and 28–30. For the i.p. immunization regimen mice were inoculated on Days 1, 14, and 28. Blood samples

were collected after puncturing the retro-orbital plexus while feces samples were collected overnight on Days 1, 13, 27 and 42. Individual blood samples of each mice group were tested for anti-LTB antibody response, pooled, and then stored at −20 °C for further ELISA tests. Fecal materials were first freeze-dried and, then, stored at −20 °C. Before testing 15 fecal pellets (approximate 0.6 g) were homogenized in 500 μl of PBS and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatants were collected and pooled for determination of LTB-specific IgA titers.

2.7. Detection of antigen-specific serum and mucosal antibody responses

Anti-LTB antibody responses were measured in 96-well MaxiSorp (Nunc) ELISA plates coated with the GM1 ganglioside (2 μg/ml in PBS buffer) and left at 25 °C for 4 h. Purified LT toxin (0.5 μg per well) was added to the plates and incubated for 2 h. After a blocking step with 1% BSA in PBS buffer for 1.5 h at 37 °C, the plates were incubated for 1 h with serially diluted mouse sera or fecal extracts diluted in PBS buffer containing 0.1% BSA plus 0.05% Tween-20. After a second washing step, plates were incubated with diluted peroxidase-conjugated rabbit anti-mouse IgG or IgA (Sigma) for 1.5 h at 37 °C. The plates were developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) and H_2O_2 and the reactions were stopped after addition of 2 M H_2SO_4. Absorbance at 492 nm was measured on a microtiter plate reader (LabSystem). All tested samples were assayed in duplicated wells. Absorbance values of pre-immune sera or sera from non-immunized mice were used as reference blanks. Dilution curves were drawn for each sample and end-point titers, represented by the means ± S.E., were calculated as the reciprocal values of the last dilution with an optical density of 0.1.

2.8. Toxin neutralization effect of anti-LTB antibodies

Determination of the in vitro LT neutralization activity of antibodies generated in mice immunized with recombinant *B. subtilis* strains were carried out in competitive GM1-ELISA. Briefly, LT aliquots (10 ng) incubated with different dilutions of the tested serum samples (100 μl in 0.1% BSA containing PBS) were incubated in microtiter plates for 1 h at room temperature. The mixtures were transferred to previously prepared microtiter plates (Maxisorp, Nunc) previously coated overnight with the GM1 ganglioside (2 μg/ml in PBS buffer) and blocked with 1% BSA in PBS buffer for 1 h at 37 °C. The plates were incubated at room temperature for 1 h and then washed three times with PBS buffer containing 0.1% BSA plus 0.05% Tween-20. An anti-CT rabbit serum diluted 1:5000 in 0.1% BSA in PBS buffer was added to the plates and incubated for 1 h at room temperature. Following three additional washing steps, a peroxidase-conjugated goat anti-rabbit IgG (final dilution 1:3000) was added and plates were incubated for 1.5 h at 37 °C. Color reactions were developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) and H_2O_2 and the reactions were stopped after addition of 2 M H_2SO_4. Absorbance at 492 nm was measured on a microtiter plate reader (LabSystem). Anti-LT neutralization titers values were determined as the reverse of the lowest serum dilutions reducing 50% of the reaction absorbance, as compared to the values obtained with pre-immune sera.

2.9. Statistical analysis

Antibody titers and standard deviations were calculated with the Microcal Origin 6.0 Professional program. The Students *t*-test was applied in comparisons of mean antibody titer values of different mouse groups. Differences with *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Episomal expression system for B. subtilis

We reasoned that the immunogenicity of *B. subtilis* cells or spores could be enhanced after delivery of higher antigen loads encoded by a multicopy episomal expression system. Additionally, we assumed that gene expression under in vivo conditions, as those found in phagosomes [24] or in the gastrointestinal tract, would further enhance immunogenicity of recombinant antigens delivered by *B. subtilis* spores or cells. Thus, we constructed a *B. subtilis* episomal multicopy expression systems based on the *E. coli–B. subtilis* shuttle vector pMTLBS72, reported to replicate via a structural stable theta mechanism in *B. subtilis* [10], and the *gsiB* gene promoter, recognized by σB and activated under different stress conditions [11,12,22].

The *gsiB* promoter region was amplified from the *B. subtilis* WW02 genomic DNA and inserted into the unique *Bam*HI site of pMTLBS72, resulting in the expression vector named pLDV1. In a second step, the DNA fragment encoding the LTB structural sequence was cloned in front of the *gsiB* promoter, resulting in the final recombinant expression vector designated pLDV2 (Fig. 1). Expression of the encoded antigen was determined in the *B. subtilis* LDV2 strain cultivated either at 28 °C or after a heat-shock at 45 °C for 2 h. As shown in Fig. 2, LTB expression was higher in cells submitted to heat-stress, but it also occurred in lower degree in cells incubated at 28 °C representing approximately one fifth of the protein produced in heat-stressed cells, probably reflecting the activation of the *gsiB* promoter during the onset of the stationary phase [23]. The amount of LTB expressed by pLDV2-transformed *B. subtilis* cells was calculated in immunoblots as 50 ng per 10^8 cells after the temperature increase, thus corresponding to 15 μg of antigen per dose administered via the p.o. route or 1 μg of antigen per dose administered via the i.p. route. No recombinant protein was detected in spores prepared from cultures incubated either at 28 °C or 45 °C (data not shown).

Fig. 2. Detection of LTB expressed by the recombinant *B. subtilis* LDV2 strain. A total of 20 μg of whole extract protein was applied per lane, proteins were separated by SDS–PAGE, electro-blotted to nitrocellulose membranes and the bands reactive with the anti-LT serum were developed with mouse anti-LT serum. Samples: 1, *B. subtilis* LDV1 cultivated at 45 °C for 2 h; 2, *B. subtilis* LDV2 cultivated at 28 °C up to the end of the exponential phase (culture OD_{600nm} 1.4); 3, *B. subtilis* LDV2 incubated at 45 °C for 2 h (culture OD_{600nm} 2.2); 4, Purified LT isolated from ETEC H10407 strain. The positions and molecular weights of LTA (30 kDa) and LTB (11.5 kDa) subunits are indicated on the right side of the figure.

3.2. Stable in vitro and in vivo gene expression mediated by pLDV2

To evaluate the stability of the recombinant pLDV2 vector in *B. subtilis* we detected the presence of the plasmid containing cells after incubation under in vitro (growth in LB) or in vivo conditions (in feces of mice orally dosed with cells or spores). As indicated in Fig. 3A, negligible segregation

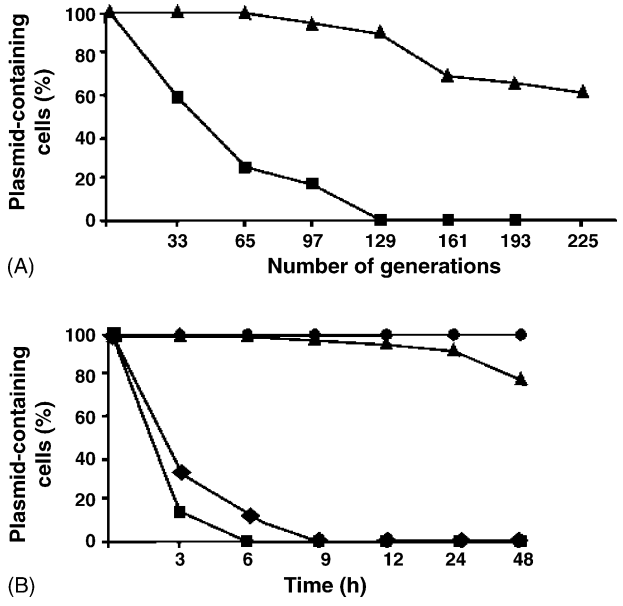

Fig. 3. Stability of *B. subtilis* expression vectors during in vitro and in vivo conditions. (A) Stability of pLDV2 (LDV2 strain) (▲) and pREP9 (LDV3 strain) (■) in *B. subtilis* after growth in LB at 37 °C without addition of chloramphenicol. Samples were harvested every 24 h (representing 33 generations) and plated on neomycin-containing medium followed by replica plating on chloramphenicol-containing plates. (B) Stability of pLDV2 (▲, ●) and pREP9 (■, ♦) in *B. subtilis* cells recovered from feces of mice inoculated with a single dose of 10^{10} spores (●, ♦) or 3×10^{10} vegetative cells (▲, ■). The numbers of Cm^r colonies were expressed as percentages of the total number of tested colonies.

was detected in pLDV2-containing cells during the first 90 generations of cultures prepared in LB. At the end of the observation period, corresponding to 225 generations, 60% of the tested colonies still harbored the recombinant plasmid. Additionally, all tested colonies were able to synthesize the heterologous antigen, as evaluated by Western blots (data not shown). In comparison, another *B. subtilis* expression vector, pREP9, reported to replicate via a single-stranded DNA intermediate [16], showed a rather reduced in vitro stability. As shown in Fig. 3A, following 129 generations in LB at 37 °C all tested colonies had lost the resistance marker encoded by the expression vector.

Plasmid pLDV2 stability was also evaluated among *B. subtilis* cells recovered from mice orally inoculated with a single dose of bacteria or spores. All colonies recovered from feces of mice dosed with vegetative cells harbored the plasmid during the first 12 h, while 80% of the cells still kept the pLDV2 expression vector 48 h after the oral dosing (Fig. 3A). All tested colonies carrying the pLDV2 vector were proficient in LTB expression (data not shown). Additionally, all *B. subtilis* cells recovered from feces of animals dosed with the LDV2 strain maintained the plasmid for at least 48 h (Fig. 3B). For comparison, we also evaluated the in vivo stability of pREP9 under in vivo conditions. All cells recovered from mice 6 h after the oral administration of cells or spores of the *B. subtilis* strain transformed with pREP9 had lost the chloramphenicol resistance marker (Fig. 3B). These results indicate that episomal expression vector, pLDV2, replicates and expresses the encoded antigen in transformed *B. subtilis* cells in rather stable fashion both under in vitro and in vivo situations as compared to the pREP9 vector.

3.3. Serum anti-LTB antibody responses elicited in mice immunized with recombinant B. subtilis bacteria or spores via parenteral and mucosal routes

The immunogenicity of the LDV2 strain was evaluated in C57BL/c mice after i.p. or p.o. inoculations of either vegetative cells or spores and the antibody responses were measured 2 weeks later. As shown in Fig. 4A, mice i.p. immunized with a single dose of 2×10^9 live *B. subtilis* vegetative cells incubated at 45 °C developed anti-LT serum IgG titers of approximately 1.1×10^4. More importantly, mice immunized with a single dose of spores derived from the *B. subtilis* LDV2 strain developed an anti-LTB titer of 7.7×10^3, as compared to mice immunized with non-recombinant spores, suggesting that LTB expression had occurred during the in vivo spore transit.

Analysis of the anti-LTB serum IgG responses in mice p.o. inoculated with a single dose consisting of three daily doses of bacteria or spores confirmed that, similar to parenterally immunized mice, serum anti-LTB IgG responses were induced both with vegetative cells incubated at 45 °C (average IgG titer of 1.1×10^3) and spores (average IgG titer of 5×10^2) (Fig. 4B). These results further indicated that antigen expression occurred during in vivo transit of *B. subtilis*

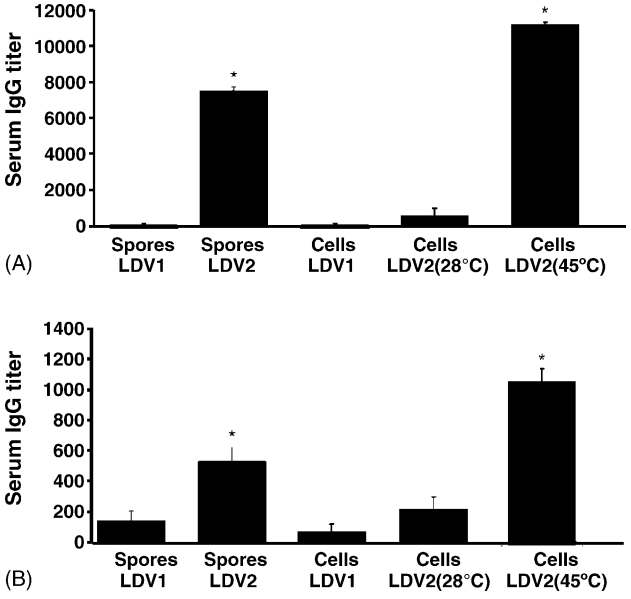

Fig. 4. LTB-specific serum IgG responses elicited in C57BL/6 mice immunized with spores or vegetative cells of *B. subtilis* LDV1 or LDV2 strains delivered via parenteral or oral routes. (A) Mice immunized via the i.p. route with a single dose of spores (10^9 CFU) or vegetative cells (2×10^9 CFU) of the *B. subtilis* LDV1 or LDV2 strains. (B) Mice immunized via the p.o. route with three consecutive daily doses of spores (1.5×10^{10} CFU) or vegetative cells (3×10^{10} CFA) of the *B. subtilis* LDV1 or LDV2 strains. The LDV2 strain was previously incubated at 28 °C until onset of stationary phase or heat-shocked at 45 °C. Blood samples were harvested 2 weeks after the last immunization. End-point titers were calculated as the reverse values of the last dilution with an optical density of 0.1.

LDV2 since no antigen was expressed by the in vitro prepared spores. No statistically significant LTB-specific IgG response was detected in mice orally inoculated with *B. subtilis* LDV2 cells cultivated at 28 °C.

Previously tested oral immunization regimens based on recombinant *B. subtilis* strains as live vaccine vectors employed three daily consecutive injections repeated three times at 2 week intervals, a procedure adapted to counterbalance the low immunogenicity of *B. subtilis* spores and vegetative cells [2,4]. We repeated the same immunization regimen based on three doses given on three consecutive days employing vegetative cells or spores of the *B. subtilis* LDV2 strain and, as shown in Fig. 5A, the induced serum IgG responses varied according to the antigen vehicle used. Mice orally immunized with vegetative cells incubated at 45 °C induced increased LTB-specific serum antibody responses with maximal IgG titer values achieved 2 weeks after the last dose (serum IgG titer of 4×10^3). On the other hand, mice immunized with *B. subtilis* spores developed peak anti-LTB responses 2 weeks after the second immunization dose (serum IgG titer of 1.5×10^3) (Fig. 5A).

3.4. Anti-LTB secreted antibody responses elicited in mice immunized with the recombinant B. subtilis LDV2 strain

Secreted LTB IgA responses were also detected in mice orally immunized with *B. subtilis* LDV2 cells or spores (Fig. 5B). All mice p.o. immunized with vegetative cells incubated at 45 °C or spores developed LTB-specific fecal IgA responses with peak antibody levels detected 13 days after the second set of doses (average IgA titers of 2.3×10^2 and 3.3×10^2 for mice immunized with spores or vegetative cells, respectively) (Fig. 5B). After the third set of doses, the specific anti-LTB IgA response levels dropped by approximately one-third to one-half as compared to values recorded in mice with two doses of spores or vegetative cells, respectively.

3.5. Analysis of IgG subclass responses immunized with recombinant B. subtilis

The LTB-specific IgG subclass (IgG1 and IgG2a) responses elicited in mice immunized with recombinant *B. subtilis* spores and vegetative cells were measured in serum samples collected from animals inoculated via i.p. or p.o. immunizations routes. As indicated in Fig. 6, mice immunized via the p.o. route, both with spores or 45 °C incubated cells, developed a prevailing type 1 response as indicated

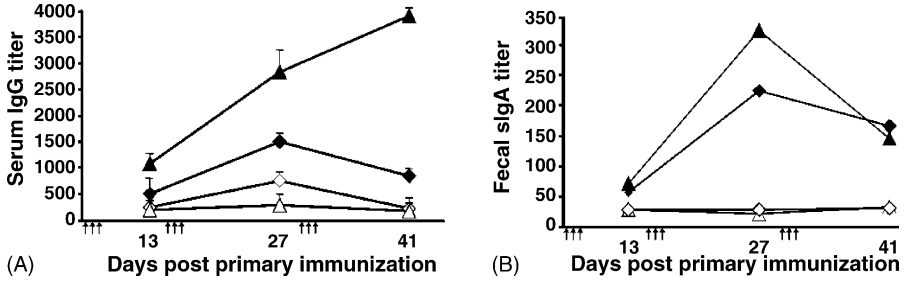

Fig. 5. Induction of the LTB-specific serum IgG (A) and fecal IgA (B) responses elicited in mice p.o. immunized with spores or vegetative cells of *B. subtilis* LDV1 or LDV2 strains. Groups of five mice were immunized with three series of three consecutive daily doses containing 3×10^{10} CFU of 45 °C-incubated vegetative cells or 1.5×10^{10} spores of the recombinant *B. subtilis* LDV2 strain or the LDV1 strain. Samples tested: *B. subtilis* LDV1 spores (◊) and vegetative cells (△); *B. subtilis* LDV2 spores (♦) and vegetative cells (▲). The arrows indicate each immunization. End-point titers were calculated as last dilution reverse values with an optical density of 0.1. *Statistically significant differences ($P < 0.005$) with regard to corresponding samples collected from mouse groups immunized with the LDV1 strain.

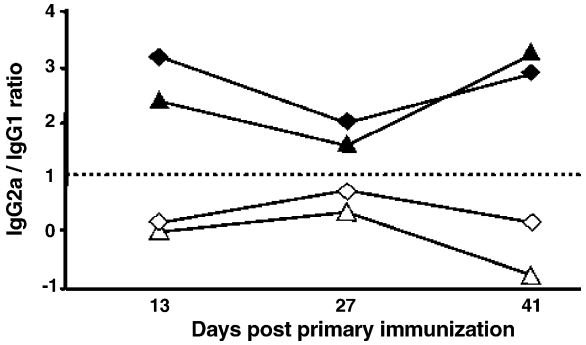

Fig. 6. Serum anti-LTB IgG subclasses responses elicited in mice immunized with spores or vegetative cells incubated at 45 °C of the *B. subtilis* LDV2 strain via i.p. or p.o. routes. Serum samples were harvested on different days post immunization from mice immunized via the i.p. route (open symbols) or p.o. route (filled symbols) with spores (diamonds) or vegetative cells (triangles) of the *B. subtilis* LDV2 strain. Mice immunized via the i.p. route received three doses at Days 1, 14, and 28 while mice submitted to the p.o. immunization regimen received doses at Days 1–3, 14–16, and 28–30. The ratio of IgG2a/IgG1 for each tested serum sample is indicated according to the time schedule of the immunization regimens.

by the predominant IgG2a subclass responses. On the other hand, mice immunized with spores or vegetative cells via the i.p. route expressed higher anti-LTB IgG1 levels, an indicative of a prevailing type 2 response (Fig. 6).

3.6. Neutralization of native LT by antibodies raised in mice immunized with recombinant B. subtilis strains

Anti-LT serum IgG antibodies raised in mice orally immunized with spores or cells of the recombinant *B. subtilis* LDV2 strain were able to recognize native LT produced by ETEC strains and block the adhesion of the toxin to the GM1 receptor. Incubation of purified LT aliquots (10 ng) with sera collected from mice immunized with spores or cells of the LDV2 strain blocked the GM1 binding activity of LT, as demonstrated in GM1-ELISA plates (Table 1). The same results were also observed when anti-LT sera raised in mice immunized with purified toxin were tested. In contrast, serum samples from mice orally immunized with cells or spores of the LDV1 strain did not any significant inhibition of LT binding in GM1-ELISA tests.

Table 1
Anti-LT neutralization titers of serum collected from mice immunized with recombinant *B. subtilis* strains

Immunization regimen[a]	IgG-ELISA titer ± S.E.[b]	LT neutralization titer[c]
Pre-immune sera	0	0
LDV1 cells	70 ± 5	0
LDV2 cells	1100 ± 145	2.85
LDV1 spores	108 ± 19	0
LDV2 spores	680 ± 86	2.65
LT	5.5×10^5	8.25

[a] Tested serum samples were harvested from BALB/c mice orally immunized with consecutive daily doses containing 3×10^{10} CFU of vegetative cells incubated at 45 °C or 1.5×10^{10} spores of the recombinant *B. subtilis* LDV2 strain (transformed with the LTB subunit encoding plasmid vector) or the LDV1 (transformed with the empty vector). A control group was immunized with four doses of purified LT (20 μg/dose) with Freund adjuvant.

[b] Means of serum IgG titer ± S.E. of serum pools harvested from the different mouse groups (minimum of five mice per group) determined in GM1-ELISA.

[c] Serum dilutions leading to a 50% reduction of the absorbance values achieved in reactions carried out with 10 ng of purified LT in GM1-ELISA assays (approximately 2.8 OD units at 492 nm).

4. Discussion

In addition to the economic relevance for industrial production of enzymes and fermented foods [25], *B. subtilis* strains have also been used as a probiotic in Europe and Asia [3]. *B. subtilis* strains have also found applications on the production of vaccines either as antigen cellular factories or as antigen carriers delivered via oral or parenteral routes [26–28]. The proposed use of *B. subtilis* spores as oral delivered vaccine vehicles has attracted considerable attention due to the extended shelf life and endogenous probiotic effect for both humans and animals [26–28]. Nonetheless, the widespread use of *B. subtilis* strains as delivery vector has been questioned by the reduced immunogenicity of both spores and cells particularly after delivery via mucosal routes. This limitation may be attributed to the brief transit of *B. subtilis* through the mammalian gastrointestinal tract and low antigen loads resulting in reduced targeting to mucosal immune responses afferent sites and defective antigen processing and presentation by antigen presenting cells.

In this work we report the development of a new *B. subtilis* antigen expression approach based on stable multicopy episomal system under the control of a stress-inducible promoter, resulting in enhanced systemic and secreted antibody responses in mice immunized with either spores or live vegetative *B. subtilis*. Under such conditions we managed to increase the immunogenicity of LTB expressed as an intracellular antigen by a recombinant *B. subtilis* strain when delivered via parenteral and oral routes. Based on this expression system, significant secreted and systemic specific antibody responses were achieved with only one dose (three consecutive daily doses) of spores or cells delivered via the oral route, representing one third of the immunization load previously reported with *B. subtilis* spores genetically modified to express heterologous antigens fused to spore coat proteins [1,2,4].

The generation of episomal expression systems capable of promoting enhanced antibody responses to antigens carried by recombinant *B. subtilis* strains involved fundamentally two steps: first, the development of an adequate episomal vector and second, the identification of a promoter allowing antigen expression duing in vivo conditions. Production of recombinant proteins encoded by episomal expression vectors is usually unstable in *B. subtilis,* as well as in other Gram-positive bacterial species, since most convenient expression vectors are derived from rolling-circle replicating plasmids

originally isolated from *S. aureus* strains, such as pUB110 [30], pC194 [31], and pE194 [30]. Although these plasmids replicate stably in *B. subtilis*, addition of recombinant DNA sequences trigger intramolecular recombination events in the single-stranded DNA intermediate that may result in deletions affecting the recombinant gene [8,29]. Such observations led to the development of integrative expression systems in which an expression cassette sandwiched between the two halves of a non-essential gene, such as *amyE* and *lacA,* is cloned in non-replicative vectors and, then, inserted into the *B. subtilis* chromosome [32,33]. The development of stable expression vectors became feasible after the discovery and handling of a *B. subtilis* bi-directional replicating plasmid allowing the development of structurally stable expression vectors [10,34].

Another relevant aspect on the development of the *B. subtilis* expression system was the selection of the *gsiB* promoter, reported to be expressed at low levels by exponentially growing cells and further activated following exposure to different stress factors such as heat, nutrient starvation, anaerobiosis and low pHs [12,22]. The cloning of the LTB toxin subunit under the control of the *gsiB* promoter resulted into a expression system that proved to be stable both under in vitro and in vivo conditions as compared to pREP9, an expression plasmid replicating via a single-stranded intermediate [16]. Moreover, the encoded peptide could be detected both in exponential phase cells incubated at elevated temperatures and in stationary phase cells deprived of nutrients. Collectively, the observations cited above indicate that the new *B. subtilis* expression system, while not requiring addition of expensive inducers, allows the expression of recombinant proteins in *B. subtilis* at a high and stable fashion, two relevant features of strains to be used either as antigen factory or delivery vectors.

Immunization of C57BL/c mice with vegetative *B. subtilis* spores revealed that three consecutive daily p.o doses were sufficient to trigger enhanced serum IgG responses to the vaccine antigen. Analysis of the induced immune responses achieved with vegetative *B. subtilis* revealed that a minimum load of pre-formed antigen is important for the activation of specific antibody responses. *B. subtilis* LDV2 cells incubated at 45 °C, but not those incubated at 28 °C expressing approximately one-fifth of the protein produced by heat-shocked cells, were able to trigger specific anti-LT antibody responses in mice immunized via the oral route. These results suggest that vegetative cells do not efficiently promote de novo protein synthesis during in vivo transit, an expected consequence of the massive cell death and lack of gut epithelium colonizing ability of *B. subtilis* [35]. In contrast, mice immunized with *B. subtilis* LDV2 spores elicited systemic and secreted anti-LTB antibody responses even after a single dose delivered either via the parenteral or oral route. The induction of specific antibody responses in mice immunized with recombinant spores further indicates that *B. subtilis* spores germinate during the transit through the gastrointestinal tract or after ingestion by phagocytic cells [24,35]. Moreover, based on the induction of LTB-specific immune responses, we conclude that the *gsiB* promoter is active under in vivo conditions, particularly at sites involved in the generation of immune responses such as GALT afferent sites and phagocytic cells.

Determination of IgG subclass responses elicited in mice immunized with the recombinant LDV2 strain suggested that both the immunization route and the nature of the bacterial carrier affect at different extents the activation of specific T helper cell populations. Although mice immunized via the parenteral route elicited higher serum IgG2a responses than those orally dosed with the same antigen carriers, mice immunized with spores were capable of mounting higher IgG2a/IgG1 ratios than those immunized with vegetative cells. These results suggest that parenteral administration and antigen delivery by recombinant spores preferentially activate type 1 responses and, consequently, trigger more efficient T cell-dependent immune responses, a feature presently under investigation with antigens derived from other pathogens.

We could also show that LTB-specific antibodies raised in mice immunized with recombinant LDV2 cells or spores were able to recognize and neutralize the receptor-binding function of LT purified from ETEC strains. This result demonstrates that, although intracellularly accumulated, LT produced by recombinant *B. subtilis* is expressed in a conformation compatible with the generation of antibodies capable of recognizing the toxin and inhibiting the receptor-binding function. Future studies should address the question if antigen expression at different cellular compartments can affect the immunogencity and epitope affinity of antibodies targeted to antigens expressed by recombinant *B. subtilis* strains.

In contrast to previous reports based on the use of recombinant *B. subtilis* spores [2,4], we observed that mice immunized with spores or vegetative *B. subtilis* LDV2 cells showed a reduction in the secreted and systemic specific antibody responses following repeated immunizations. It is conceivable that the repeated antigen exposure carried by *B. subtilis* strain would lead to immunological tolerance due to the large number of immunization doses and amount of delivery cells or spores. We believe that the development of improved *B. subtilis* antigen expression systems and establishment of optimized immunization regimens would enhance the immunogenicity of passenger antigens and the more efficient use of *B. subtilis* strains as vaccine vehicles.

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