Evaluation of different promoter sequences and antigen sorting signals on the immunogenicity of Bacillus subtilis vaccine vehicles

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Abstract

Recombinant Bacillus subtilis strains, either in the form of spores or vegetative cells, may be employed as safe and low-cost vaccine vehicles. In this study, we studied the role of promoter sequences and antigen-sorting signals on the immunogenicity based on previously constructed B. subtilis episomal expression systems. Mice orally immunized with spores or cells encoding the B subunit of the heat labile toxin (LTB), originally expressed by some enterotoxigenic Escherichia coli (ETEC) strains, under control of the stress-inducible gsiB promoter developed higher anti-LTB serum IgG and fecal IgA responses with regard to vaccine strains transformed with plasmids encoding the antigen under control of IPTG-inducible (Pspac) or constitutive (PlepA) promoters. Moreover, surface expression of the vaccine antigen under the control of the PgsiB promoter enhanced the immunogenicity of vegetative cells, while intracellular accumulation of LTB led to higher antibody responses in mice orally immunized with recombinant B. subtilis spores. Specific anti-LTB antibodies raised in vaccinated mice recognized and neutralized in vitro the native toxin produced by ETEC strains. Nonetheless, only mice orally immunized with recombinant B. subtilis strains, either as vegetative cells or spores, expressing intracellular LTB under the control of the gsiB promoter conferred partial protection to lethal challenges with purified LT. The present report further demonstrates that B. subtilis plasmid-based heterologous protein expression systems are adequate for antigen delivery via the oral route.

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1. Introduction

Bacillus subtilis strains have found different applications during the last decades including the industrial production of proteases, preparation of alkaline-fermented food and as a probiotic product for prevention of enteric infections both in humans and animals [1–3]. Additionally, the knowledge on genetics and physiology of B. subtilis together with the development of appropriate genetic tools have brought further interest in the use of genetically modified B. subtilis strains for the expression of pharmaceutically useful recombinant proteins and antigen carrier vehicles in vaccine formulations [4–6]. The use of B. subtilis spores as vaccine vehicles is particularly appealing since, due to the natural resistance to heat and different environmental stresses [7], recombinant spores are easily prepared and maintained at room temperature dramatically reducing production and storage costs. Indeed, bacterial spores can remain dormant and viable for immense periods of time, perhaps millions of years [8].
The initial step towards the use of genetically engineered *B. subtilis* spores as vaccine vehicles was based on the surface expression of recombinant antigens genetically fused to spore coat proteins [9–11]. Based on a single copy chromosomally integrated gene expression system, the tetanus toxin fragment C (TTFC) was expressed at approximately $10^3$ copies per recombinant spore [9]. In such an “out model”, the pre-formed antigen is expressed at the surface of recombinant spores but de novo synthesis would not be expected to occur during passage through the host gastrointestinal environment. Oral or intranasal administration of recombinant *B. subtilis* spores to mice resulted in significant, but at low levels, specific secreted and systemic antibody responses to the encoded antigen and conferred protection to lethal doses with the whole purified toxin [10,11].

Using a different “in” approach, our group described the development of an alternative gene expression system allowing the intracellular production of recombinant antigens exclusively by vegetative *B. subtilis* cells transformed with an episomal vector endowed with enhanced structural and segregation stability [12]. Moreover, antigen expression under the control of a stress-inducible Sigma B-dependent promoter allowed expression during in vivo transit through the vertebrate host, as demonstrated by the induction of antigen-specific systemic and secreted antibodies following oral administration of spores to mice [12] supporting the notion that ingested *B. subtilis* spores germinate during the transit through the host organism [13–15]. However, the impact of different promoter sequences and sorting signals on the immunogenicity of antigens encoded by recombinant *B. subtilis* strains using such antigen expression system remains unchecked.

The recent construction of new *B. subtilis* episomal expression vectors allowing production of recombinant proteins under the control of different promoters and carrying sorting signals for secretion [16] or cell wall anchoring [17] offers the possibility to determine the in vivo performance of orally delivered recombinant *B. subtilis* strains as vaccine vehicles. In this study, we describe the immunogenicity of *B. subtilis* vaccine strains encoding the B subunit of the heat-labile toxin (LTB), originally expressed by some entero-toxigenic *Escherichia coli* strains, an important etiologic agent of infant and traveler’s diarrhea [18]. Based on the present results, the influence of promoter activity and antigen-sorting signals on the induced antibody response elicited in mice orally vaccinated with recombinant *B. subtilis* strains could be inferred.

### 2. Materials and methods

#### 2.1. Bacterial strains and growth conditions

All *B. subtilis* and *E. coli* K12 strains used in the present study are described in Table 1. Bacterial strains were routinely cultivated in Luria-broth (LB) with added neomycin (25 µg/mL) and/or chloramphenicol (5 µg/mL) and erythromycin (1 µg/mL), for *B. subtilis*, or ampicillin (100 µg/mL) for *E. coli*, as appropriate. *B. subtilis* strains was induced in Difco-sporulation media (DSM) using the exhaustion method as previously described [19]. *E. coli* competent cells were prepared with the CaCl₂-mediated transformation protocol [20], while *B. subtilis* competent cells were obtained by the two-step transformation method [21].

#### 2.2. Plasmid constructions

Amplification of the LTB-encoding gene (*eltB*), derived from genomic DNA of the ETEC H10407 strain, was carried out with primers ELTBFW and ELTB2Rv, as previously described [12]. Once digested with *Bgl*II and *Xba*I, the amplified fragment was cloned into the vectors pHCMC03, pHCMC02 and pHCMC05 [16], treated with *Bam*HI and *Xba*I restriction enzymes. The resulting recombinant plasmids were named pLDV5, pLDV8 and pLDV11 and encoded the LTB subunit under the control of the stress inducible Sigma B-dependent promoter derived from the gsiB gene, the constitutive promoter derived from the lepA gene, and the IPTG-inducible promoter derived from the *spa* gene, respectively [16]. All expression plasmids were derived from pHCMC03 [16], which differ from our previously reported vector [12] by the presence of the transcriptional terminator *trpA* ensuring the efficient termination of transcription immediately downstream of the recombinant genes. One recombinant plasmid of each construction was

<table>
<thead>
<tr>
<th>Bacterial strains used in the present study</th>
<th>Main characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em> WW02</td>
<td>leuA8 metB3 trpC2 hsdRI M1 amyE::neo</td>
<td>[27]</td>
</tr>
<tr>
<td><em>B. subtilis</em> NHD03</td>
<td>WW02 with srtA gene under control of Pspac</td>
<td>[16]</td>
</tr>
<tr>
<td><em>B. subtilis</em> LDV4</td>
<td>WW02 carrying pHCMC03</td>
<td>This work</td>
</tr>
<tr>
<td><em>B. subtilis</em> LDV5</td>
<td>WW02 carrying pLDV5 (<em>eltB</em> under control of PgsiB)</td>
<td>This work</td>
</tr>
<tr>
<td><em>B. subtilis</em> LDV8</td>
<td>WW02 carrying pLDV8 (<em>eltB</em> under control of Pspac)</td>
<td>This work</td>
</tr>
<tr>
<td><em>B. subtilis</em> LDV11</td>
<td>WW02 carrying pLDV11 (<em>eltB</em> under control of Pspac)</td>
<td>This work</td>
</tr>
<tr>
<td><em>B. subtilis</em> LDVsecr2</td>
<td>WW02 carrying pLDVsecr2 (secretion of LTB with α-amyrase amylQ signal sequence under control of PgsiB)</td>
<td>This work</td>
</tr>
<tr>
<td><em>B. subtilis</em> LDVanc2</td>
<td>NHD03 carrying pLDVanc2 (anchoring of LTB in the cell wall under control of PgsiB)</td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>recA1 endA1 gyrA96 glnV44 = supE44 relA1 deoR Δ(lacZ-argF)U169 hsdR17 thi-1 λ− φ80d lacΔ(lacZ)M15 F−</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
first subjected to restriction enzyme analysis, then nucleotide sequencing and, finally, introduced into *B. subtilis* WW02. The secretion vector was generated following amplification of the coding region for the signal peptide of the *B. amyloliquefaciens* *amyQ* gene using specific primers (AmyISFBcII, 5′-GGCCATTTGATCATATGGAAAAA CGAAGCGGAGAC 3′ and AmyIPSRBamHI, 5′-GGCCATGATGCTTCATCGG 3′, restriction sites underlined) and plasmid pNDHI5 [17] as template. The amplified fragment (93 bp) was subsequently cloned into the BamHI-cleaved pHCMC03 vector carrying the PgsiB promoter [16]. The resulting plasmid containing the insert in the right orientation was named pLDVsecr1. The final cloning step involved amplification of the eltB gene with primers ELTBFw and ELTB2Rv and cloning of the resulting fragment, after digestion with BglII and XbaI, into pLDVsecr1 cleaved with the same restriction enzymes. One recombinant plasmid, named pLDVsecr2, was selected, subjected to restriction analysis, nucleotide sequencing and introduced into the *B. subtilis* WW02. The expression vector allowing anchoring of recombinant LTB to the *B. subtilis* cell wall peptidoglycan cross bridges was obtained following amplification of the 3′ terminal end of the *S. aureus fnbB* gene with specific primers (FnbBAtIII, 5′-GGCCATTGACGTCGCGGAATG GTAACCAATCATTCGAAGAAG 3′ and FnBrBSsuI, 5′-GGCCATAGGCGCTTATGCTTTTG GTAATCTTTTATTCTC GC 3′) and plasmid pNDH21 [17] as template. The amplified fragment was cloned into the pLDVsecr1 digested with AarII and StuI resulting in pLDVanc1. The eltB gene, encoding 103 residues of the complete LTB subunit amino acid sequence, was amplified using primers ELTBFw and ELTBRB (5′-GGCCATGATGCTTCATCGG 3′) and, following digestion with BglII and BamHI, cloned into BamHI-cleaved pLDVanc1. The final vector allowed the recombinant protein to be expressed with a 196 amino acid spacer region after the peptidoglycan anchor sequence [17]. One recombinant clone, carrying the plasmid named pLDVanc2, was selected, subjected to restriction analysis, nucleotide sequencing and introduced into the *B. subtilis* NHD03, encoding the *Listeria monocytogenes* sortase A under the control of the spac promoter, as previously described [17].

2.3. In vitro expression of LTB by recombinant *B. subtilis* strains

Detection of recombinant LTB expressed by *B. subtilis* strains was achieved after cultivation in Erlenmeyer flasks aerated in an orbital shaker set at 200 rpm at 37°C overnight. New cultures were prepared after diluting cells (1:100) into fresh LB medium kept at 37°C under aeration until an OD600nm of 0.6–0.8 was reached. IPTG was added to a final concentration of 0.1 mM at this point in cultures prepared with the LDV11 strain (activation of the Pspac required for expression of L. monocytogenes sortase A required for cell wall anchoring of LTB). Cultures prepared with LDV5 (activation of the PgsiB promoter leading to intracellular accumulation of LTB), LDVsecr2 (activation of the PgsiB promoter leading to secretion of LTB) and LDVanc2 (activation of the PgsiB promoter leading to surface expression of LTB) strains were heat shocked at 45°C for 2 h. No special treatment was applied to cultures of the *B. subtilis* LDV8 strain (intracellular production of LTB protein under the control of PlepA). Whole cell extracts were prepared after incubation of cells to a final OD600nm of approximately 2.2 and suspension of the cells in lysis buffer as described before [12]. Release the cell wall-anchored LTB expressed from pLDVanc2 was achieved by treating cells with lysozyme (500 μg/ml dissolved in water) for 30 min at room temperature [17]. Released proteins were precipitated with trichloroacetic acid [20]. Quantitative estimates of LTB produced by each recombinant *B. subtilis* strain were carried out using Western blots of whole cell extracts at established cell densities or culture supernatants, mouse anti-LT serum and determined quantities of purified LT produced by recombinant *E. coli* strains.

2.4. SDS-PAGE and Western blots

SDS-PAGE was performed following standard procedures using 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% (w/w) β-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 [22]. Western blots were carried out following incubation of nitrocellulose sheets with a mouse polyclonal anti-LT antiserum and development of reactive bands with a chemoluminescent kit (Super Signal, Pierce), as previously described [12].

2.5. Preparation of spores

Sporulation of *B. subtilis* was induced in DSM using the exhaustion method as previously described [19]. Viable spores were titrated for determination of the number of CFU/ml and then transferred to −20°C until use.

2.6. Immunization regimens

C57BL/6 and BALB/c female mice were supplied by the isogenic mouse breeding facility of the Department of Immunology, Biomedical Sciences Institute (ICB), University of Sao 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 via the oral (p.o.) route with cells or spores of the *B. subtilis* strains LDV5, LDV8, LDV11,
LDVsec2 or LDVanc2. Suspensions (0.5 ml aliquots) containing approximately $3 \times 10^{10}$ vegetative cells or $1.5 \times 10^{10}$ spores were delivered with a stainless-steel round tip gavage cannule at days 1–3, 14–16, and 28–30. Mice received 0.5 ml of a 0.1 M sodium bicarbonate solution 30 min before the administration of the bacteria or spores. Blood and fecal samples were collected three days before the immunization regimen and on days 13, 27 and 44. Individual blood samples of each mice group were tested for the anti-LTB antibody response, pooled, and then stored at $-20\,^\circ\mathrm{C}$ for further testing. Fecal materials were first freeze-dried and stored at $-20\,^\circ\mathrm{C}$ and processed as previously described [12].

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

Anti-LTB antibody responses were measured by GM1-ELISA carried out in 96-well MaxiSorp (Nunc) plates coated with the GM1 ganglioside according to conditions described before [12]. 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 serum sample and endpoint titers, represented as the reciprocal values of the last dilution with an optical density of 0.1, expressed as the means ± S.D. of all animals submitted to the same vaccine regimen. Serum LTB-specific IgG subclass responses were measured with same experimental procedure but using peroxidase-conjugated rabbit anti-mouse IgG1 and IgG2a (Pharmigen). Fecal LTB-specific IgA titers, represented by means ± S.E., were measured in pooled fecal pellets recovered from mouse groups submitted to the same vaccine regimen.

2.8. Determination of plasmid stability under in vivo conditions

Plasmid stability under in vivo conditions was measured in groups of five female mice inoculated with a single 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 analyzed time points. Sets of 5–10 chloramphenicol resistant colonies were also submitted to Western blot experiments to evaluate LTB expression.

2.9. Toxin neutralization effects of anti-LTB antibodies

Determination of the in vitro LT neutralization activity of LT-specific antibodies was achieved by competitive GM1-ELISA. Briefly, LT aliquots (10 ng) incubated with different dilutions of the tested serum samples were incubated in microtiter plates for 1 h at room temperature and transferred to microtiter plates (Maxisorp, Nunc) previously coated with the GM1 ganglioside. Blocking, incubation with rabbit anti-cholera toxin (CT)-specific antibodies and color reaction steps were performed exactly as previously described [12]. Absorbance at 492 nm was measured on a microtiter plate reader (LabSystem) and the anti-LT neutralization titers were determined as the reverse of the lowest serum dilution yielding a 50% reduction of the absorbance measured with toxin samples incubated with pre-immune sera.

2.10. Toxin challenge experiments

Groups of four female C57BL/6 mice immunized with nine oral dose sets of recombinant *B. subtilis* strains either in the form of vegetative cells (LDV4, LDV 5 and LDVanc2) or spores (LDV5) were challenged i.p. with purified LT (50 μg), corresponding to two-fold the minimal lethal dose, 2 weeks following the last immunization. Mice were observed daily, and mortality rates were measured during 1 week.

2.11. Statistical analysis

Antibody titers and standard deviations were calculated with the Microcal Origin 6.0 Professional program. The Student 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. *B. subtilis* vaccine vehicles allowing antigen expression under the control of different promoters and accumulation at different cellular compartments

The development of a new series of *B. subtilis* expression vectors conferring stable production of recombinant proteins under the control of different promoters was the starting point for the construction of vaccine strains expressing LTB intracellularly under the control of two inducible (PgstB in LDV5 and Pspac in LDV11) and one constitutive promoter (PlepA in LDV8) [16]. Moreover, based on construction of expression vectors encoding the signal sequence of *B. subtilis* α-amylase and the anchoring motif of the *S. aureus* fibronectin-binding protein B (FnbB) and the *L. monocytogenes* sortase A (SrtA) [17], we investigated the impact of cell-sorting signals on the immunogenicity of LTB encoded by *B. subtilis* vaccine vehicles (LDVanc2 and LDVsec2 strains), either in the form of vegetative cells or spores. A schematic linear representation of constructed episomal expression vectors, expected size and amount of the encoded products are shown in the Fig. 1.
The monitoring of LTB production during in vitro growth of the *B. subtilis* LDV5, LDV8 and LDV11 strains revealed that PgsiB conferred the highest antigen expression by vegetative cells following induction at 45°C for 2 h (Fig. 2A). Under such conditions, the amount of LTB accumulated by *B. subtilis* cells reached approximately 30 ng in 10^8 CFU, representing an increase of 5- to 10-fold, as compared to values obtained with cells incubated at 37°C. As expected, the amount of LTB produced under control of the constitutive PlepA did not change during in vitro cultivation of the LDV8 strain and reached values of approximately 10 ng in 10^8 CFU. Finally, the *B. subtilis* LDV11 strains (Pspac) accumulated the LTB antigen at concentrations ranging from 5 to 10 ng in 10^8 CFU and the amounts of LTB detected in cells cultivated in the presence of IPTG for 2 h was approximately two-fold higher than the amount of antigen detected in cultures prepared without the inducer (Fig. 2A). As also expected, no recombinant antigen was detected in spores produced by each of the three tested *B. subtilis* strains (data not shown). Based on the higher protein yields obtained with the LDV5, further experiments aiming the evaluation of antigen sorting signals on the immunogenicity of *B. subtilis* were based on the PgsiB promoter.

Targeting LTB to the extracellular environment was achieved in two different ways. The N-terminal in-frame fusion of the α-amylase signal sequence coding region to LTB allowed the secretion of the antigen into the extracellular medium using LDVsecr2, while co-expression of *L. monocytogenes* sortase A and the C-terminal in-frame fusion of the *S. aureus* FnB anchoring motif allowed surface expression of the antigen by *B. subtilis* LDVanc2 cells. As shown in Fig. 2B, immunoblot analyses of secreted protein fractions of the LDVsecr2 strain confirmed that LTB accumulated in the growth medium of cultures prepared either at 37 or 45°C. Curiously, the secreted protein cross-reacting with the LT-specific serum had an apparent electrophoretic mobility of 57 kDa, instead of the expected 11.5 kDa of the B subunit monomer, suggesting oligomerization of the encoded protein subunit that, for unknown reasons, was resistant to the SDS-PAGE denaturing conditions. Lysozyme-soluble fractions recovered from the *B. subtilis* LDVanc2 incubated in the presence of IPTG revealed the presence of protein bands with diverse molecular weights cross-reacting with the LT-specific serum corresponding to the protein attached to peptidoglycan fragments with different lengths and partial proteolysis of the encoded peptide. As expected, no residual LTB was detected in spore preparations carried out with strains LDVanc2 and LDVsecr2 strains (data not shown).

All tested recombinant *B. subtilis* strains cultivated in vitro for 100 generations retained the LTB-encoding plasmid as
monitored by the presence of the chloramphenicol resistance marker. Moreover, the number of chloramphenicol-resistant colonies recovered from feces 48 h after oral administration of the vaccine strains to mice ranged from 88 to 100%, while all colonies recovered from mice fed with B. subtilis spores retained the expression vectors (data not shown).

3.2. Evaluation of systemic and secreted immune responses in mice after oral administration of LTB-expressing B. subtilis strains

As indicated in Fig. 3, the immunogenicity induced by strain LDV5, applied either as vegetative cells or spores, was higher than those achieved by LDV8 and LDV11 as demonstrated by the amount of LTB-specific serum IgG or fecal IgA, thus, indicating that the stress-inducible promoter derived from the gsiB gene has a better in vivo performance than the IPTG-inducible (Pspac) and the constitutive (PlepA) promoters. As previously observed [12], the systemic and secreted immune responses elicited in mice orally immunized with B. subtilis vegetative cells were consistently higher (maximal mean IgG titer of 1005 ± 110 and fecal IgA titer of 214 in animals immunized with LDV5 cells) than those elicited in mice inoculated with spores (maximal mean IgG titer of 450 ± 70 and fecal IgA titer of 100 in animals immunized with LDV5 spores) (Fig. 3).

In another series of experiments, we evaluated the relevance of cell sorting signals on the immunogenicity of B. subtilis vaccine vehicles. Surface expression of LTB in LDVanc2 cells enhanced by at least two-fold the serum IgG anti-LTB responses (mean IgG titer of 2000 ± 450) in mice immunized with B. subtilis LDV5 spores) (Fig. 3).
Mice immunized with LDVsecr2 cells or spores did not elicit any significant serum IgG or fecal IgA LTB-specific responses (Fig. 3). Taken together, these results indicate that expression of LTB anchored at the cell wall enhanced the serum antibody responses in mice immunized with vegetative cells. Nonetheless, the intracellular expression of LTB under the control of PgsiB proved to be the better approach to enhance systemic and secreted antibody responses in mice orally dosed with recombinant spores.

The serum IgG responses elicited in mice orally dosed with \textit{B. subtilis} LDV5 and LDVanc2 strains were followed at different time points during the immunization regimen. As indicated in Fig. 4, mice immunized with one, two or three dose sets of LDVanc2 cells elicited consistently higher anti-LTB serum IgG responses than mice immunized with vegetative cells of the LDV5 strain. In contrast, mice orally dosed with LDV5 spores elicited higher anti-LTB serum IgG responses than those inoculated with the LDVanc2 strain irrespective of the dose number (Fig. 4).

The LTB-specific serum IgG subclass responses were analyzed in mice immunized with spores or vegetative cells of the \textit{B. subtilis} LDV5 and LDVanc2 strains. As indicated in Fig. 5, mice orally immunized with vegetative cells of either LDV5 or LDVanc2 strains developed a more biased Th1 response as inferred by the predominant serum IgG2a subclass response. Indeed, the IgG2a/IgG1 ratios detected in mice immunized with \textit{B. subtilis} LDV5 or LDVanc2 strains increased according to the number of immunization doses reaching a IgG2a/IgG1 ratio close to 6 after the third dose set. In contrast, mice orally immunized with spores of LDV5 and LDVanc2 strains developed a more balanced Th1/Th2 response with IgG2a/IgG1 ratios ranging from 1 to 2 (Fig. 5). On the other hand, mice inoculated parenterally (i.p.) with both cells and spores of both vaccine strains developed a pronounced Th2-response with IgG2a/IgG1 ratios bellow 1 (data not shown).

### 3.3. LTB-specific antibodies raised in mice immunized with \textit{B. subtilis} vaccine vehicles neutralize LT produced by ETEC strains

Anti-LTB serum antibodies raised in mice orally dosed with spores or vegetative cells of the \textit{B. subtilis} LDV5 or LDVanc2 strains recognize native LT produced by ETEC strains. Incubation of purified LT with sera harvested from mice orally immunized with cells or spores of the \textit{B. subtilis} LDV5 or LDVanc2 strains blocked the binding of LT to the GM1-ganglioside receptor, as demonstrated in GM1-ELISA (Table 2). Similar results were obtained with LT-specific serum raised in i.p. mice immunized with the native protein purified from ETEC cells. No significant inhibition of LT receptor-binding function was recorded with mouse sera harvested from mice immunized with cells or spores of the \textit{B. subtilis} LDV4 strain (Table 2).

### 3.4. Challenge experiments

The vaccine potential of the LTB-specific antibodies raised in C57/BL6 mice immunized with recombinant \textit{B. subtilis} strains was evaluated following a lethal parenteral challenge with purified LT extracted from ETEC cells. As shown in Fig. 6, mice immunized with cells of the \textit{B. subtilis} LDV4 strain did not survive the challenge with LT. Similarly, no mice immunized with \textit{B. subtilis} LDVanc2 cells survived
of different B. subtilis recently described plasmids [16,17] conferring segregation-clones[4,6,10]. In this study, we evaluated the immunogenicity heat-resistant, and low-cost mucosal delivered vaccine vehicle renewed interest on the development of safe, easily prepared, safely engineered to express vaccine antigens has brought the LDV5 strain, either as spores or vegetative cells, conferred the challenge with purified LT. However, immunization with LDV4 cells, (■) LDV5 cells, (▲) LDV5 spores or (▲) LDVanc2, the elicited antibody response towards LTB, used as model antigen.

The antigen load of a vaccine vehicle, either biotic or abiotic, is a key parameter affecting immunogenicity in mammals, particularly following delivery via the oral route. Among Gram-positive bacteria, the unstable expression of recombinant proteins encoded by single strand intermediate replicating plasmids led to the adoption of expression systems based on integration of the target gene at non-essential loci on the bacterial chromosome, thus reducing the gene copy number and the amount of accumulated antigen [9,11,23]. The construction of B. subtilis theta-like replicating expression plasmids endowed with structural and segregational stability permitted the accumulation of higher antigen loads and enhanced the immunogenicity of spores and vegetative cells [16,17,24]. Our estimates indicated that maximal antigen loads, reaching up to 9 µg of antigen per dose were achieved in expression vectors carrying the gsiB promoter following a temperature upshift. Curiously, the amount of antigen expressed by B. subtilis strains transformed with derivatives of pHCMC03 encoding LTB was reduced when compared to values previously achieved with a previous expression vector based on the gsiB promoter [12]. So far we do not have a clear explanation for such difference since the only difference between the two expression vectors is the presence of the trpA transcriptional terminator placed at the end of the cloned heterologous gene in pHCMC03 [12,16]. In both cases the in vivo expression of the target antigen occurs during traffic through the gastrointestinal tract, as demonstrated by the generation of specific antibody responses in mice immunized with spores. Indeed, several environmental conditions favoring activation of PgsiB were expected to be faced both by vegetative cells and spores including the low-stomach pH, anaerobic environments, restricted carbohydrate supply and the presence of an oxidative environment in phagosomes of macrophages and other antigen-presenting cells [25]. Considering that B. subtilis spores are able to germinate during the transit through the gastrointestinal tract [13–15], the gsiB promoter, as well as other Sigma-B dependent stress-inducible genes, seems particularly interesting for oral immunization purposes with live vaccine vehicles leading to higher antigen loads in B. subtilis vegetative cells and more efficient expression following spore germination.

Surface expression of antigens has been frequently claimed to enhance the immunogenicity of Gram-positive

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Table 2

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

<table>
<thead>
<tr>
<th>Immunization regimen</th>
<th>Anti-LTB IgG-ELISA (titer ± S.E.)</th>
<th>LT neutralization titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-immune sera</td>
<td>0 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>LDV4 cells</td>
<td>99 ± 10</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>LDV5 cells</td>
<td>1005 ± 110</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>LDVanc2 cells</td>
<td>2500 ± 100</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>LDV4 spores</td>
<td>300 ± 20</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>LDV5 spores</td>
<td>450 ± 70</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>LDVanc2 spores</td>
<td>200 ± 200</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>LT</td>
<td>5.5 x 10^5 ± 780</td>
<td>8.25 ± 0.5</td>
</tr>
</tbody>
</table>

a Serum samples collected from mice immunized with three dose sets of the recombinant spores or cells of the B. subtilis LDV5 and LDVanc2 vaccine strains. A pre-immune serum sample and sera from mice immunized with LT purified from ETEC cells were used as negative and positive control samples, respectively.

b Mean IgG titers ± S.E. of serum pools collected from mouse groups submitted to the same immunization regimens. IgG titers determined in GM1-ELISA, as described in the text.

c Serum dilutions causing a 50% reduction of the absorbance values reached in reactions with 10 ng of purified LT in GM1-ELISA tests (approximately 2.8 OD units at 492 nm).

d Hyper immune serum raised in mice C57BL/6 immunized s.c. with four doses of purified LT (10 µg) with complete (first two doses) or incomplete (last two doses) with a final IgG2a/IgG1 of 0.02 or less.

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4. Discussion

The recent demonstration that B. subtilis spores can be safely engineered to express vaccine antigens has brought renewed interest on the development of safe, easily prepared, heat-resistant, and low-cost mucosal delivered vaccine vehicles [4,6,10]. In this study, we evaluated the immunogenicity of different B. subtilis vaccine strains transformed with a set recently described plasmids [16,17] conferring segregationally/structurally stable expression of heterologous proteins under the control of different promoter sequences (PlepA, PgsiB and Pspsac), and allowing secretion or cell-wall anchoring of the target antigen. Supporting our previous evidences [12], the stress-inducible gsiB gene promoter had a better performance in vivo, both in vegetative cells and spores, than the other tested promoters, as measured by the induction of systemic and secreted specific antibody responses. Moreover, anchoring the vaccine antigen to the cell wall had enhanced the immunogenicity of orally delivered B. subtilis vegetative cells but reduced the in vivo neutralization activity of the elicited antibody response towards LTB, used as model antigen.
vaccine vehicles [26]. The present data indicated that surface exposure of the encoded antigen enhances the immunogenicity of B. subtilis LDVanc2 vegetative cells. However, due to limitations of the tested expression vector, sortase expression would not be expected to occur in the absence of IPTG and, therefore, would restrict the interpretation of results obtained in mice immunized with recombinant spores. Nonetheless, anchoring of the target antigen to the cell wall of vegetative cells resulted in statistically significant enhancement (p < 0.05 when compared to the IgG levels achieved in mice immunized with LDV5 cells) of the serum IgG anti-LTB titers. On the other hand, secretion of the encoded antigen did not result in significant induction of LTB-specific immune responses in mice inoculated with recombinant cells. A finding that may reflect either the low amount of encoded antigen or the degradation by extracellular proteases. However, taking into account the in vivo LT neutralizing activity of the induced LTB-specific antibodies, neither secretion nor cell wall anchoring seems to improve the vaccine potential of the elicited serum IgG responses.

Characterization of the IgG subclass responses of mice orally immunized with B. subtilis LDV5 or LDVanc2 cells revealed a prevailing type 1 immune response either with spores or vegetative cells, as measured by the serum IgG2a/IgG1 subclass ratios. The higher LTB-specific IgG2a/IgG1 ratios (close to 6) recorded in mice immunized with vegetative cells of LDV5 and LDVanc2 as compared to our previous reported data might be attributed to the slightly reduced antigen load expressed by these strains compared to the previously used strain [12]. Indeed, based on our present and previous data, definition of antigen-specific IgG2a/IgG1 subclass ratio in mice immunized with B. subtilis strains is more closely related to the administration route than to the nature of the vaccine vehicle or antigen cellular location. Slightly different serum IgG subclass responses obtained with B. subtilis spores reported by other groups might be ascribed to the nature of the encoding antigen or the use of the intranasal administration route [6,10,11,28].

The present results showed that anti-LTB antibodies raised in mice immunized with cells and spores of the recombinant B. subtilis LDV5 and LDVanc2 vaccine strains recognize and neutralize in vitro the receptor-binding activity of native LT isolated from ETEC. Nonetheless, in vivo experiments demonstrated that mice immunized with LDVanc2 cells did not survive parenteral challenges with the native toxin. Although LT produced by human-derived ETEC strains did not exert any toxic effects to orally challenge mice, the i.p. administration of the toxin has been shown to kill adult mice, thus representing a protection correlate based on the toxin neutralization properties of the induced serum antibody response [29]. On the other hand, half of the mice immunized with spores or cells of the LDV5 strain were protected to the lethal challenge strongly suggesting that the induced LTB-specific response efficiently recognize and neutralize the native toxin. Collectively, these evidences indicate that intracellular expression of LTB, in contrast to the same protein genetically fused to the cell wall of B. subtilis, probably preserves conformational epitopes required for the generation of surface-exposed domains involved with recognition of host cell receptors and, thus, represents the best approach to express the antigen by recombinant vaccine vectors.

Experimental vaccines targeting diarrhea caused by ETEC strains usually rely on the induction of secreted IgA responses to colonization factor antigens and LT [22,30]. The present results indicated that the recombinant B. subtilis LDV5 strain, either in the form of spores or vegetative cells, elicit both systemic IgG and secreted IgA response to LTB. Considering the potential probiotic effects of B. subtilis spores [31], the development of vaccine vehicles carrying antigens of enteric pathogens represents an interesting alternative for immediate therapeutic intervention and long term induction of protective immune responses. Nonetheless, considerable improvements in the performance of B. subtilis-based vaccine vehicle involving both immunogenicity and specificity of the induced immune responses should be pursued before efficient antigen expression systems could effectively be used in the development of new vaccine vehicles for enteric and systemic infections of humans and animals.

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