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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 (P*spac*) or constitutive (P*lepA*) promoters. Moreover, surface expression of the vaccine antigen under the control of the P*gsiB* 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.

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

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].

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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 enterotoxigenic *Escherichia coli* strains, an important ethiologic 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/m) and/or chloramphenicol (5 μg/ml) and erythromycin (1 μg/ml), for *B. subtilis*, or ampicillin (100 μg/m) for *E. coli*, as appropriate. Sporulation of the *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 $CaCl_2$-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*III and *Xba*I, the amplified fragment was cloned into the vectors pHCMC03, pHCMC02 and pHCMC05 [16], treated with *BamH*I 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 *spac* 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 1
Bacterial strains used in the present study

Bacterial strains	Main characteristics	Source
B. subtilis WW02	*leuA8 metB5 trpC2 hsrdRM1amyE::neo*	[27]
B. subtilis NHD03	WW02 with *srtA* gene under control of P*spac*	[16]
B. subtilis LDV4	WW02 carrying pHMC03	This work
B. subtilis LDV5	WW02 carrying pLDV5 (*eltB* under control of P*gsiB*)	This work
B. subtilis LDV8	WW02 carrying pLDV8 (*eltB* under control of P*lepA*)	This work
B. subtilis LDV11	WW02 carrying pLDV11 (*eltB* under control of P*spac*)	This work
B. subtilis LDVsecr2	WW02 carrying pLDVsecr2 (secretion of LTB with α-amylase *amyQ* signal sequence under control of P*gsiB*)	This work
B. subtilis LDVanc2	NHD03 carrying pLDVanc2 (anchoring of LTB in the cell wall under control of P*gsiB*)	This work
E. coli DH5α	*recA*1 *endA*1 *gyrA*96 *glnV*44 = *supE*44 *relA*1 *deoR* Δ(*lacZ-argF*)U169 *hsdR*17 *thi*-1 λ^- φ80d*lac* Δ(*lacZ*)M15 F^-	Invitrogen

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 (AmylSFBclI, 5′-GGCCATTGATCAATGATTCAAAAA CGAAAGCGGACAG 3′ and AmylPSRBamHI, 5′-GGCCATGGATCCTACGGCTGAT GTTTTTGTAATCGG 3′, restriction sites underlined) and plasmid pNDH15 [17] as template. The amplified fragment (93 bp) was subsequently cloned into the *Bam*HI-cleaved pHCMC03 vector carrying the P*gsiB* 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 *Bgl*II and *Xba*I, 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 (FnbBAatII, 5′-GGCCATGACGTCCCGCGGAATG GTAACCAATCATTCGAAGAAG 3′ and FnbBRStuI, 5′-GGCCATAGGCCTTTATGCTTTG TGATTCTTTTTATTTCTGC 3′) and plasmid pNHD21 [17] as template. The amplified fragment was cloned into the pLDVsecr1 digested with *Aat*II and *Stu*I 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′-GGCCATGACGTCTTAGGATCCGTTTTCCATACTGATTGCCGC 3′) and, following digestion with *Bgl*II and *Bam*HI, cloned into *Bam*HI-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 OD_{600nm} 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 P*spac* leading to the intracellular accumulation of LTB) and the LDVanc2 strain (activation of P*spac* required for expression of *L. monocytogenes* sortase A required for cell wall anchoring of LTB). Cultures prepared with LDV5 (activation of the P*gsiB* promoter leading to intracellular accumulation of LTB), LDVsecr2 (activation of the P*gsiB* promoter leading to secretion of LTB) and LDVanc2 (activation of the P*gsiB* 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 P*lepA*). Whole cell extracts were prepared after incubation of cells to a final OD_{600nm} 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 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 via the oral (p.o.) route with cells or spores of the *B. subtilis* strains LDV5, LDV8, LDV11,

LDVsecr2 or LDVanc2. Suspensions (0.5 ml aliquots) containing approximately 3×10^{10} vegetative cells or 1.5×10^{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 °C for further testing. Fecal materials were first freeze-dried and stored at −20 °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 (LDV 5) 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 (P*gsiB* in LDV5 and P*spac* in LDV11) and one constitutive promoter (P*lepA* 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.

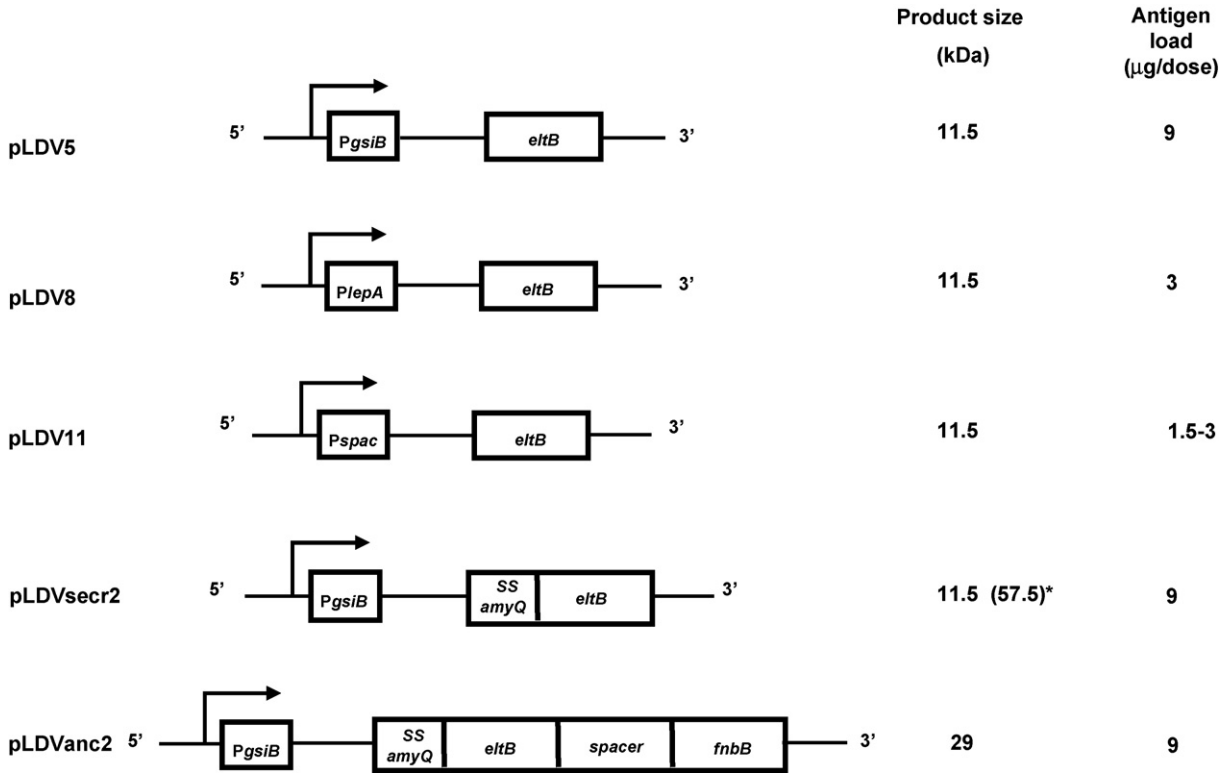

Fig. 1. Schematic linear representation of the genetic organization of the pLDV5, pLDV8, pLDV11, pLDVsecr2 and pLDVanc2 plasmids predicted molecular weight and estimated amounts of the encoded products. The amounts of recombinant protein in each immunization dose (3×10^{10} CFU) encoded by each construct were estimated in Western blots using known concentrations of LT or LTB previously purified from *E. coli* strains. Plasmids pLDV5, pLDV8 and pLDV11 encode LTB protein in the cytoplasm of transformed *B. subtilis* strains under the control of *gsiB*, *lepA* and *spac* promoters, respectively. (*) A single protein band with molecular weight of 57 kDa, corresponding to putative pentamer of the LTB subunit, was consistently detected in culture supernatants of *B. subtilis* strains transformed with the pLDVsecr2 vector.

The monitoring of LTB production during in vitro growth of the *B. subtilis* LDV5, LDV8 and LDV11 strains revealed that P*gsiB* 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 P*lepA* 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 (P*spac*) 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 P*gsiB* 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* FnbB 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

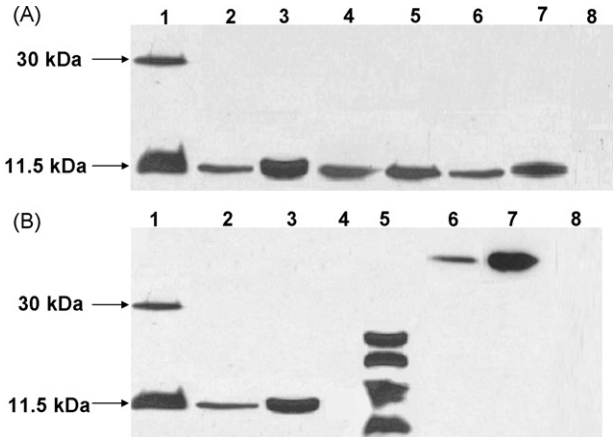

Fig. 2. Detection of in vitro expressed LTB encoded by the recombinant *B. subtilis* strains. (A) Expression of LTB under the control of different promoters. Lane 2, LDV5 (P*gsiB*) strain incubated at 37 °C. Lane 3, LDV5 (P*gsiB*) strain incubated at 45 °C. Lane 4, LDV8 (P*lepA*) strain at early exponential phase. Lane 5, LDV8 (P*lepA*) strain at late exponential phase. Lane 6, LDV11 (P*spac*) strain incubated without IPTG. Lane 7, LDV11 (P*spac*) strain incubated in the presence of 0.1 mM IPTG for 2 h; lane 8 LDV4 (negative control) strain. (B) Expression of LTB in different cell compartments. Lane 2, LDV5 strain incubated at 37 °C. Lane 3, LDV5 strain incubated at 45 °C. Lane 4, lysozyme-digests containing cell wall-anchored proteins of the LDVanc2 strain incubated without IPTG; Lane 5, lysozyme-digests containing cell wall-anchored proteins of the LDVanc2 strain incubated with 0.1 mM IPTG for 2 h, Lane 6, LDVsecr2 strain culture supernatant following incubation at 37 °C. Lane 7, LDVsecr2 strain culture supernatant following incubation at 45 °C; lane 8, LDV4 (negative control) strain. Whole cell extracts (approximately 20 μg of total protein) were probed with anti-LT mice serum and developed in Western blots with peroxidase-conjugated rabbit anti-mouse serum. Purified LT (0.1 μg) was applied in Lane 1 of both panels. Positions and molecular weight of the (A) 30 kDa and (B) 11.5 kDa subunits of LT are indicated.

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 (P*spac*) and the constitutive (P*lepA*) 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

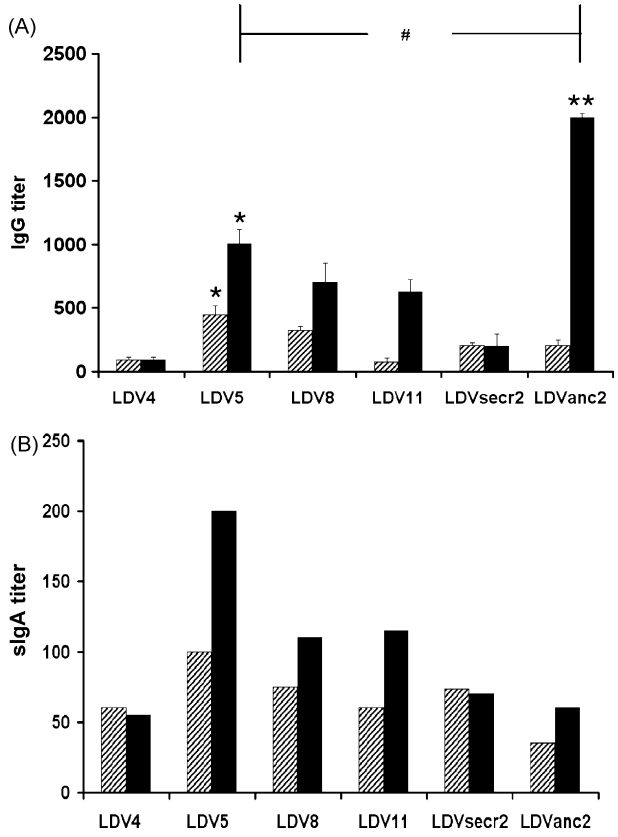

Fig. 3. Systemic and secreted antibody responses elicited in mice immunized with *B. subtilis* strains encoding LTB under the control of different promoters and sorted to different cellular compartments. LT-specific serum IgG (A) or fecal IgA (B) titers were measured in C57BL/6 mice immunized via the p.o. route with three consecutive doses of spores (hatched columns) or cells (black columns). Mice were immunized with the recombinant *B. subtilis* LDV4 (negative control strain), LDV5 (intracellular LTB expression under the control of P*gsiB*), LDV8 (intracellular LTB expression under the control of P*lepA*), LDV11 (intracellular LTB expression under the control of P*spac*), LDVsecr2 (secreted LTB expression under the control of P*gsiB*) or LDVanc2 (cell wall-anchored LTB expressed under the control of P*gsiB*). All serum IgG and fecal IgA values were measured 2 weeks following the last immunization dose. Values corresponding to serum IgG titers represent the means ± S.D. of two independent experiments with five animals per immunization group. Fecal IgA (means ± S.E.) results are measured in fecal pools collected from each mice group. Statistically significant differences at $p < 0.05$ (*) or $p < 0.005$ (**) with regard to values recorded in mice immunized with LDV4 strain and with regard to values achieved with LDVanc2 and those recorded in mice immunized with LDV5 at $p < 0.05$ ($^{\#}$).

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 ± 100) detected in mice immunized with *B. subtilis* LDV5 cells. In contrast, the immunogenicity of LDV5 spores was superior to that detected in mice inoculated with spores of the LDVanc2 strain, as measured by the LTB-specific serum IgG titers.

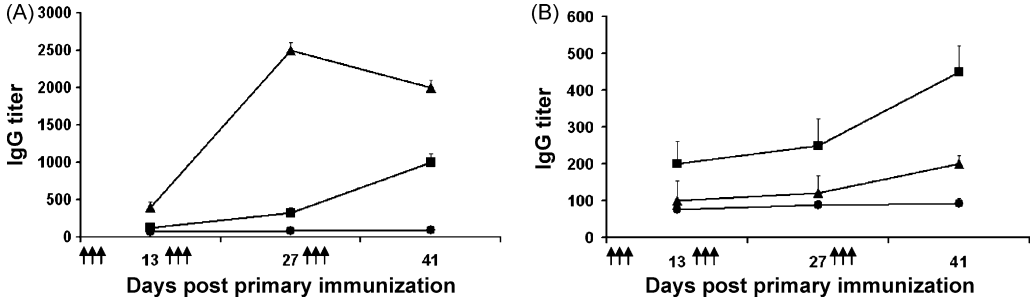

Fig. 4. Kinetics of the anti-LT serum IgG responses elicited in mice orally immunized with vegetative cells or spores of the *B. subtilis* LDV5 or LDVanc2 strains. Induction of LT-specific serum IgG responses elicited in C57BL/6 female mice orally immunized with vegetative cells (A) or spores (B). Mice were immunized with one, two or three series of three daily doses of the *B. subtilis* LDV4 (●) LDV5, (■) or LDVanc2, (▲) vaccine strains. The arrows indicate on which days a vaccine dose was administered. Titer values are represented by the mean ± S.D. of five animals per immunization group.

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 P*gsiB* 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 *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 *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 *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).

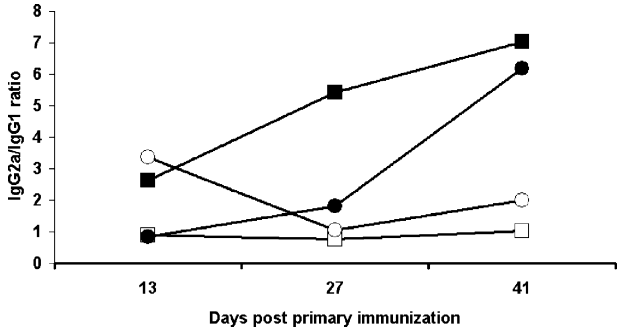

Fig. 5. Serum IgG subclass responses in orally immunized mice with spores or cells of the *B. subtilis* LDV5 and LDVanc2 strains. Serum samples were collected at different time periods during the immunization regimen from animals receiving spores (open symbols) or cells (closed symbols) of the *B. subtilis* LDV5 (circles) or LDVanc2 (squares) vaccine strains. The IgG2a/IgG1 ratios of each tested serum sample are indicated according to the immunization periods and nature of the vaccine strains.

3.3. LTB-specific antibodies raised in mice immunized with 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 *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 *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 *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 *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 *B. subtilis* LDV4 strain did not survive the challenge with LT. Similarly, no mice immunized with *B. subtilis* LDVanc2 cells survived

Table 2
Anti-LT neutralization titers of serum samples collected from mice immunized with recombinant *B. subtilis* strains

Immunization regimen[a]	Anti-LTB IgG-ELISA (titer ± S.E.)[b]	LT neutralization titer[c]
Pre-immune sera	0	0
LDV4 cells	99 ± 10	0
LDV5 cells	1005 ± 110	2.8
LDVanc2 cells	2500 ± 100	3.1
LDV4 spores	109 ± 20	0
LDV5 spores	450 ± 70	2.6
LDVanc2 spores	200 ± 23	1.3
LT[d]	$5.5 \times 10^5 \pm 780$	8.25

[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.

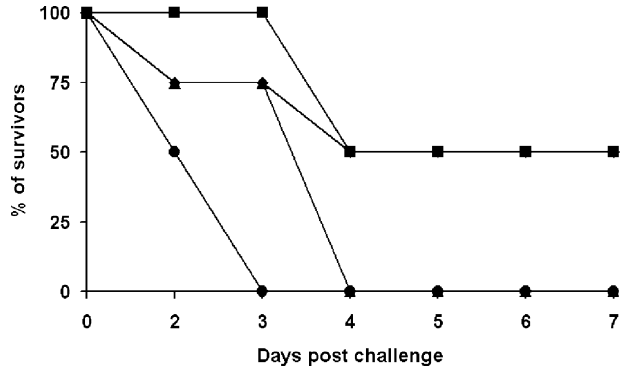

Fig. 6. Challenge of C57BL/6 mice immunized with recombinant *B. subtilis* strains with purified LT. Groups of four mice were i.p. challenged with 50 μg of purified LT toxin 2 weeks following the last oral immunization with different *B. subtilis* vaccine strains, as indicated by the symbols: (●) LDV4 cells, (♦) LDV5 cells, (■) LDV5 spores or (▲) LDVanc2.

the challenge with purified LT. However, immunization with the LDV5 strain, either as spores or vegetative cells, conferred 50% protection to mice challenged with LT.

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 (P*lepA*, P*gsiB* and P*spac*), 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.

The antigen load of a vaccine vehicle, either biotic or abiotic, is a key parameter affecting immunogenicity in mammalians, 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 at 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 P*gsiB* 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

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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