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Boosting systemic and secreted antibody responses in mice orally immunized with recombinant *Bacillus subtilis* strains following parenteral priming with a DNA vaccine encoding the enterotoxigenic *Escherichia coli* (ETEC) CFA/I fimbriae B subunit

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ABSTRACT

Recombinant *Bacillus subtilis* strains, either spores or vegetative cells, may be employed as safe and low cost orally delivered live vaccine vehicles. In this study, we report the use of an orally delivered *B. subtilis* vaccine strain to boost systemic and secreted antibody responses in mice i.m. primed with a DNA vaccine encoding the structural subunit (CfaB) of the CFA/I fimbriae encoded by enterotoxigenic *Escherichia coli* (ETEC), an important etiological agent of diarrhea among travelers and children living in endemic regions. DBA/2 female mice submitted to the prime-boost immunization regimen developed synergic serum (IgG) and mucosal (IgA) antibody responses to the target CfaB antigen. Moreover, in contrast to mice immunized only with one vaccine formulation, sera harvested from prime-boosted vaccinated individuals inhibited adhesion of ETEC cells to human red blood cells. Additionally, vaccinated dams conferred full passive protection to suckling newborn mice challenged with a virulent ETEC strain. Taken together the present results further demonstrate the potential use of recombinant *B. subtilis* strains as an alternative live vaccine vehicle.

1. Introduction

Bacillus subtilis strains have been engineered to express different antigens and employed as live recombinant vaccine vehicles delivered via the oral route either as spores or vegetative cells [1–6]. So far, two heterologous protein expression approaches have been successfully adapted to *B. subtilis* strains as vaccine vehicles for mucosal delivery of antigens. In the first reported antigen expression method, the target protein is anchored on the surface of recombinant *B. subtilis* spores based on genetic fusions with the C terminal ends of CotB or CotC spore coat proteins [7,8]. Based on a single copy chromosomaly integrated expression system, the recombinant protein is specifically activated during the sporulation phase and the pre-formed chimeric antigen is found only at the surface of recombinant spores [1,3,4,7,8].

In a conceptually different approach, we developed a second *B. subtilis* antigen expression model adequate for oral delivered vaccine vehicles. Our system employed a multi-copy episomal expression vector derived from natural *B. subtilis* plasmids endowed with enhanced structural and segregation stability [2,9]. Based on this expression system, the recombinant protein is produced by *B. subtilis* vegetative cells under the control of a stress-inducible sigma B controlled promoter, derived from the *gsiB* gene, which is activated by environmental shifts including changes in growth temperature, pH, nutrient or oxygen content, thus, allowing expression of the vaccine antigen both by vegetative cells and germinating spores [2,9]. In animals immunized with recombinant spores, antigen expression would occur only following germination of orally delivered spores during transit through the gastrointestinal tracts or in phagolysosome in antigen presenting cells [2–4]. In both expression models, specific antibody responses have been raised in mice immunized with spores or vegetative cells via mucosal or parenteral inoculation routes [1,2,4,8,10].

Enterotoxigenic *Escherichia coli* (ETEC) strains are the main etiological agents of travelers' diarrhea and a leading cause of infantile death in developing countries [11]. ETEC patho-

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genesis relies on the ability to produce the heat-stable (ST) and/or heat-labile (LT) enterotoxins after attachment of bacteria to the intestinal epithelia by means of distinct colonization factors (CFs or CFAs) [12]. The CFA/I fimbriae is the most prevailing CF produced by ETEC strains isolated in several South America and Asian countries and has been successfully used as an target antigen in several ETEC vaccine formulations [12–14].

In a previous report, we described a CFA/I-based vaccine regimen consisting in parenteral priming with a DNA vaccine encoding the structural subunit of the CFA/I fimbriae (CfaB) followed by boosting with a recombinant *Salmonella* Typhimirium strain encoding the same antigen [15,16]. The anti-ETEC prime-boost vaccine regimen synergistically enhanced both systemic and secreted CFA/I-specific antibody responses in vaccinated mice. Moreover, the combined prime-boost vaccine regimen conferred protective responses in animals challenge with a virulent ETEC strain, representing a new vaccine strategy for the control of mucosal and systemic pathogens. However, safety concerns exist for any vaccine regimen based on attenuated pathogens such as *S. enterica*. Thus, it would be important to determine if similar protective levels could be achieved with a non-pathogenic non-invasive bacterial host employed as live oral delivered vaccine vehicle to boost systemic and secreted anti-ETEC responses.

In the present study, we evaluated the vaccine potential of recombinant *B. subtilis* strains after submitting mice to a vaccine regimen based on parenteral priming with a DNA vaccine followed by oral boosting with recombinant *B. subtilis* strains, both vaccines vehicles encoding the CfaB protein as the target antigen. The results clearly show that *B. subtilis* strains can efficiently boost systemic and secreted specific anti-ETEC antibody responses in mice previously primed with the target antigen. Additionally, anti-CfaB antibodies raised in mice submitted to the prime-boost vaccine regimen neutralized the binding properties of CFA/I$^+$ ETEC cells and conferred full passive protection to suckling neonate mice, born from vaccinated dams, challenged with loads of pathogenic bacteria. Taken together these results clearly demonstrate the potential usefulness of live recombinant *B. subtilis* strains as orally delivered vaccine vehicles.

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 [2]. The *B. subtilis* LDV6 and LDVanc3 strains were obtained after transformation of WW02 strain with the pLDV6 and pLDVanc3 expression vectors, respectively. The *B. subtilis* LDV4 strain, obtained after transformation of the WW02 strain with pHMC03 plasmid, was used as negative control strain in some immunization experiments. All manipulations involved in vector construction and cloning of the CfaB-encoding gene were carried out with the *E. coli* strain DH5α as a recipient host. Bacterial strains were routinely grown in Luria-Bertani 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*. Bacterial strains were cultivated at 30 °C (*B. subtilis*) or 37 °C (*E. coli*). The ETEC 258909-3 strain (CFA/I$^+$, O128:H?, ST/LT) and a non-fimbriated plasmid-cured derivative, 258909-3M strain, were supplied by Dr. A.M. Svennerholm (University of Gothenburg, Sweden). The ETEC strains were cultivated in casamino acids/yeast extract (CFA) agar plates at 37 °C for 18 h [15]. *E. coli* competent cells were prepared with the $CaCl_2$-mediated transformation protocol, while *B. subtilis* cells were submitted to the two-step transformation method, as previously reported [17].

2.2. Plasmid constructions

Amplification of the CfaB-encoding gene (*cfaB*), derived from genomic DNA of the ETEC 258909-3 strain, was carried out with primers CFA5Fw (5′ GAGGAAAGATCTATGGAGAAAAATATTACTGTAACAG) and CFA3Rv (5′ CGACGTCTCTAGAACTGGATCCCAAAGTCATTACAA). Once digested with BglIII and XbaI, the amplified fragment was cloned into pHCMC03 cleaved with BamHI and XbaI restriction enzymes [9]. *B. subtilis* cells transformed with the recombinant vector pLDV6 accumulate the CfaB protein into the cytoplasm under the control of the stress inducible *gsiB* gene promoter [9]. Generation of cell wall-anchoring vectors involved three cloning steps first by fusing the signal sequence of the *B. amyloliquefaciens amyQ* gene to the sequence encoding the CfaB subunit, following by subcloning into pHCM03 and, finally, cloning of the sequence corresponding to the 3′ terminal end of the cell wall anchor domain of the *S. aureus* fibronectin-binding protein (FnbB), as previously described [10,18]. The resulting expression vector, named pLDVanc3, allowed expression of the recombinant antigen genetically fused to the cell wall on the surface of vegetative cells. The *B. subtilis* vaccine strains transformed with pLDV6 and pLDVanc3, allowing expression of CfaB in the cytoplasm or exposed at the cell surface of vegetative cells, were named LDV6 and LDVanc3, while spores generated from the same strains were named sLDV6 and sLDVanc3, respectively. The DNA vaccine pRECFA encodes a membrane-anchored hybrid CfaB protein sandwiched fused with the herpes simplex virus type 1 glycoprotein D (gD) under the control of a Roux Sarcoma virus promoter [19,20]. pRECFA was purified by equilibrium density centrifugation in $CsCl_2$ gradient, repeated twice, precipitated with ethanol and suspended in PBS and DNA concentration was measured spectrophotometrically and adjusted to the final concentration of 1 mg/mL.

2.3. SDS-PAGE and Western blots

SDS-PAGE was performed following standard procedures using a Mini Protean II vertical electrophoresis unit (Miniprotean, 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 [21]. Western blots were carried out following incubation of nitrocellulose sheets with MAb84, a CfaB-specific mouse monoclonal antibody, and development of reactive bands with a chemoluminescent kit (Super Signal, Pierce).

2.4. Preparation of spores

Sporulation of *B. subtilis* was induced following cultivation in DSM broth using the exhaustion method as previously described [22]. During preparation, all spore samples were incubated at 70 °C for 40 min to eliminate any viable vegetative cells. Viable spores were titrated for determination of the number of CFU ml^{-1} and then transferred to −20 °C until use.

2.5. Preparation of the B. subtilis–ETEC vaccine strains

In vitro expression of the CfaB subunit by *B. subtilis* LDV6 and LDVanc3 strains was achieved following previously reported

conditions [10]. Induction of CfaB expression under control of the *gsiB* promoter, present in both *B. subtilis* vaccine strains, was obtained after incubation of exponentially growing cells at 45 °C for 2 h. Activation of *L. monocytogenes* sortase A, required for cell wall anchoring of CfaB encoded by the *B. subtilis* LDVanc3 strain, was achieved following addition of 0.1 mM IPTG to exponentially growing heat-shocked cells. Whole cell extracts were prepared with cells harvested at a final $OD_{600\,nm}$ of approximately 2.2. *B. subtilis* LDVanc3 was treated with lysozyme (500 μg/mL dissolved in water) for 30 min at room temperature to release the CfaB protein covalently linked to the cell wall and subsequently precipitated with trichloroacetic acid [9]. Quantitative estimates of CfaB produced by each recombinant *B. subtilis* strain were carried out using Western blots of whole cell extracts and determined quantities of purified heat denatured CFA/I fimbriae produced by *E. coli* 258909-3 strain using a CfaB-specific mouse monoclonal antibody (Mab84).

2.6. Immunization protocols and sample collection

DBA/2 mice were supplied by the Isogenic Mouse Breeding Facility of the Department of Parasitology, Biomedical Sciences Institute (ICB), University of São Paulo (USP), and all animal handling procedures were in accordance with the principles of the Brazilian code for the use of laboratory animals. Groups of five female DBA/2 mice with ages ranging from 6 to 8 weeks were primed with two intramuscular (i.m.) doses of 100 μg of pRECFA (50 μg of DNA at each hind limb tibialis anterior muscle), with an interval of two weeks, and boosted with two oral doses, each consisting of three consecutive daily doses, of *B. subtilis* vegetative cells or spores (10^{10} CFU) given in a 2-week interval, 4 weeks following the last DNA dose. Once induced for CfaB expression at 45 °C, *B. subtilis* cells were washed once by centrifugation with PBS and suspended in 0.1 M sodium bicarbonate to a final concentration of 2×10^{10} CFU/mL. Mice were immunized twice intragastrically (p.o.) with 0.5-mL aliquots of bacterial suspensions using a stainless steel round tip gavage cannule. *B. subtilis* spores were harvested from cells cultivated under non inducing conditions, suspended to the same final concentration and delivered to mice using the same procedure. Serum samples were collected before immunizations (pre-immune sera) and 2 weeks after the last inoculation with the *B. subtilis* LDV6 and LDVanc3 strain. Serum samples were individually tested for reactivity with the CfaB antigen by enzyme-linked immunosorbent assay (ELISA), pooled and stored at −20 °C for subsequent analysis. Fecal samples were collected per mouse group submitted to the same immunization procedure during one night. Fecal pellets were pooled, freeze-dried and stored at −20 °C. Fifteen pellets (approximately 0.6 g) were homogenized in PBS, centrifuged at 10,000 × *g* for 10 min at 4 °C and supernatants collected for detection of CfaB-specific IgA in ELISA.

2.7. Detection of CfaB-specific antibody responses

CfaB-specific serum and fecal antibody responses were measured in ELISA carried out with purified heat-denatured CFA/I fimbriae as a solid-phase bound antigen, according to previously described procedures [15,20]. All tested samples were assayed in duplicated wells. Results of serum and fecal extract pools were expressed as endpoint titers, represented by the means ± S.E., calculated as the reciprocal values of the last dilution with an optical density of 0.1 or twice that of the serum and fecal samples collected from pre-immune animals or mice immunized with the *B. subtilis* LDV4 strain.

2.8. Inhibition of haemagglutination assays (IHA)

Blocking of haemagglutination mediated by CFA/I-expressing ETEC cells were carried out with pooled serum samples of mice submitted to different immunization regimens, according to previously published procedures [15]. Results were evaluated by visual inspection and the maximal dilutions able to inhibit the haemagglutination reactions promoted by the ETEC 258909-3 strain were registered. The ETEC 258909-3M (CFA/I-) was used as a negative control. The assay was performed in presence of D-mannose, at concentration of 1% (w/v), to avoid unspecific binding mediated by type 1 fimbriae. The test was independently repeated at least three times for pooled samples harvested from each immunization group.

2.9. Challenge of infant mice with human-derived ETEC strains

The newborn mice were challenged with ETEC 258909-3 strain as previously described [15,16]. Aliquots of ETEC cells were previously incubated with serum pool dilutions, harvested from mice submitted to different immunization regimens, for 30 min at room temperature. Aliquots (50 μl) of the bacterial suspension, corresponding to a final concentration of 2×10^7 CFU ($20 \times LD_{50}$ for the ETEC 258909-3 strain), were inoculated into the stomachs of one or two-day-old pups with disposable syringes with ultra-thin needles (12.7 mm × 0.33 mm). Newborns died between 24 h and 6 days following the lethal challenge were considered for determination of mortality rates. Each serum samples was tested with 4–6 litters (15–20 pups/tested serum). Passive protection experiments were carried out with female DBA/2 mice submitted to the prime-boost vaccine regimen. Mice were mated at same week of the first *B. subtilis* vaccine dose and litters were delivered 10–20 days after the last *B. subtilis* dose.

2.10. Statistical analysis

Antibody titers and standard deviations were calculated with the Microcal Origin 8.0 Professional program. The Student's *t*-test was applied in comparisons of mean antibody titer values of different mouse groups and differences with *P* values below 0.05 were considered to be statistically significant.

3. Results

3.1. Construction of recombinant B. subtilis vaccine strains expressing the CfaB subunit under control of a stress-inducible promoter and sorted to different cellular compartments

B. subtilis strains have been previously shown to induce passenger antigen-specific systemic (IgG) and secreted (IgA) antibody responses in mice orally immunized with either vegetative cells or spores engineered to express the ETEC heat labile toxin *B. subunit* [2,10]. In the present study, we evaluated the performance of recombinant *B. subtilis* vaccine strains for boosting specific antibody responses in mice previously primed with a DNA vaccine encoding the structural subunit (CfaB) of the ETEC CFA/I fimbriae. For this purpose, we constructed two *B. subtilis* strains encoding the CfaB protein under control of the *gsiB* gene promoter, accumulated either in the cytoplasm (LDV6 strain) or at the cell surface covalently linked to the cell wall of vegetative cells (LDVanc3). Expression of the CfaB subunit by the *B. subtilis* LDV6 and LDVanc3 strains was detected in vegetative cells, but, as expected, not in spores, following induction at 45 °C for 2 h (Fig. 1). Similarly, no reacting protein band was detected in whole cell extract prepared

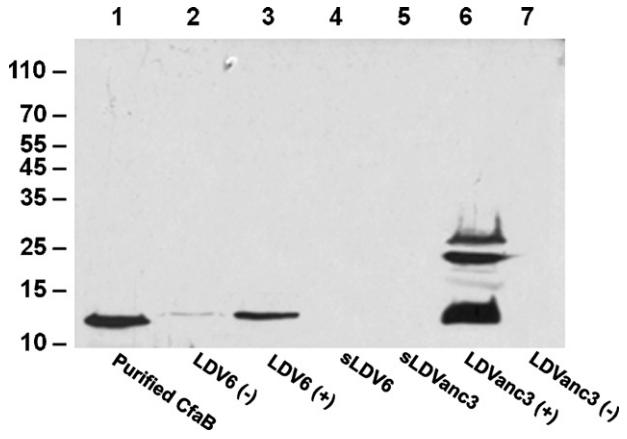

Fig. 1. Expression of CfaB by recombinant *B. subtilis* vaccine strains. Western blots were prepared with purified CfaB protein (lane 1), whole-cell extracts (lanes 2, 3, 6 and 7) or spore coat soluble material (lanes 4 and 5) of *B. subtilis* LDV6 strain (lanes 2, 3 and 5) or LDVanc3 (lanes 6–7) and developed with the CfaB-specific MAb 84. *B. subtilis* LDV6 and LDVanc3 vegetative cells were harvested under inducing (lanes 3 and 6) or no inducing condition (lanes 2 and 7). Samples corresponding to vegetative cells (LDV6, LDVanc3) harvested under inducing (+) or non-inducing (−) conditions, as well as spores (sLDV6 and sLDVanc3) are indicated in the figure. Molecular mass references (kDa) are indicated on the left side of the figure.

with the *B. subtilis* LDV4 strain, which does not carry the CfaB-encoding gene (data not shown). Surface expression of the CfaB antigen by the *B. subtilis* LDVanc3 cells required in vitro cultivation in the presence of IPTG, in order to activate expression of the *L. monocytogenes* SrtA sortase, expressed in the recombinant strain under the control of the IPTG-inducible p*spac* promoter, thus allowing the correct anchoring of heterologous protein at the cell wall by means of the genetically fused C-terminal domain of the *S. aureus* FnbB protein [10]. Monitoring of CfaB production during in vitro growth of the *B. subtilis* LDV6 and LDVanc3 strains revealed that, in both strains, the amount of CfaB reached approximately 30 ng in 10^8 CFU, representing an increase of 5- to 10-fold with regard to the amount of antigen obtained with cells incubated at 37 °C (Fig. 1 and data not shown). Additional protein bands, reacting with the anti-CfaB monoclonal antibody, were detected in lysozyme-soluble extracts of the LDVanc3 strain, probably reflecting the covalent linkage of the recombinant protein to cell wall fragments of different lengths (Fig. 1). As expected, no residual CfaB was detected in spore derived from both recombinant *B. subtilis* strains (Fig. 1). Moreover, all tested recombinant *B. subtilis* strains cultivated in vitro for 100 generations retained the CfaB-encoding plasmid as monitored by the presence of the chloramphenicol resistance marker. The same results were obtained with colonies recovered from feces of mice orally dosed 48 h before with the vaccine strains either as vegetative cells or spores (data not shown).

3.2. Generation of anti-CfaB antibody responses in mice submitted to the DNA priming-Bacillus boosting vaccine regimen

Priming DBA/2 mice with two i.m. doses of pRECFA followed by two oral boosting doses with the recombinant *B. subtilis* vaccine strains, either with spores or vegetative cells, synergistically enhanced the systemic (serum IgG) and secreted (fecal and milk IgA) antibody responses to the encoded CfaB antigen. Higher systemic IgG responses were reached in mice boosted with vegetative *B. subtilis* cells when compared to animals boosted with spores but both groups elicited systemic anti-CfaB responses significantly higher than those immunized only with pRECFA or the *B. subtilis* strains even after administration of multiple dose vaccine regimens (Fig. 2) [10,19,20]. On the other hand, animals boosted with spores of the *B. subtilis* LDV6 strain developed higher anti-CfaB fecal IgA titers than mice immunized with vegetative cells, best reasoned by the in vivo expression of the target antigen following spore germination during transit through the gastrointestinal tract. In contrast, spores of the *B. subtilis* LDVanc3 strain did not induce any measurable anti-CfaB fecal IgA responses, probably reflecting the lack of in vivo sortase A expression required for cell wall anchoring of the target antigen at the surface of the nascent cell (Fig. 2). Mice primed with pRECFA and orally boosted with the *B. subtilis* LDV4 strain did not elicit enhanced systemic or secreted anti-CfaB response with regard to mice immunized only with the DNA vaccine (data not shown).

Oral administration of *B. subtilis* spores or vegetative cells result in prevailing type 2 responses with IgG1/Ig2a ratios close to 3.5 while mice immunized parenterally with pRECFA developed a predominant type 1 response pattern (IgG1/IgG2a ratio of 0.77), as previously reported (Fig. 3) [10,19]. On the other hand, mice submitted to the DNA-prime/*B. subtilis*-boosting vaccination regimen developed a more balanced Th1/Th2 pattern with IgG1/IgG2a ratios ranging from 1.09 (mice boosted with vegetative cells of the LDVanc3 strain) to 1.45 (mice boosted with spores of the LDV6 strain) (Fig. 3).

3.3. Protective vaccine effects of anti-CfaB antibodies generated in mice submitted to the prime-boost vaccine regimen

To confer immunological protection to ETEC strains, anti-CfaB antibodies generated in mice submitted to different vaccine regimens must recognized epitopes located on the surface of intact CFA/I fimbriae and inhibit the binding of live ETEC to host cell receptors. As shown in Table 1, serum antibodies raised in mice submitted to the prime/boosting vaccine regimen neutralize the binding properties of live CFA/I$^+$ ETEC bacteria in contrast to serum samples harvested from pRECFA-vaccinated mice, as demonstrated by the ability to inhibit the haemagglutination promoted by the ETEC 258909-3 strain. The IHA titers determined in serum pools prepared from mice submitted to the prime/boost vaccine regimen ranged from 30 to 35% of the corresponding CfaB-specific IgG ELISA titers, except by the group immunized with spores of the *B. subtilis* LDVanc3 strain whose sera displayed no IHA activity (Table 1). These results indicate that the DNA priming/*B.subtilis* boosting immunization regimen enhanced both quantitatively and qualitatively, with regard to the epitope specificity toward the CFA/I fimbriae, the systemic and secreted antibody responses elicited in vaccinated mice.

Earlier experiments allowed us to establish a lethal challenge model with human-derived ETEC strain using neonate mice of the DBA/2 strain [15,16]. Based on this model, we could also demonstrate that the lethality induced by CFA/I$^+$ ETEC strains was fully neutralized by CfaB-specific antibodies recognizing epitopes present in the native CFA/I fimbriae expressed by live ETEC cells raised in mice primed with a pRECFA and boosted with attenuated *S.* Typhimurium vaccine strains [15,16]. In the present study, we applied the same approach to demonstrate the protective role of CfaB-specific antibodies generated in mice boosted with *B. subtilis* vaccine strains. Initially, CFA/I$^+$ ETEC cells of the 258909-3 strain cells were incubated with serum samples of mice submitted to different vaccine regimens before inoculation into the stomach of newborn DBA/2 mice. The results indicated that neonate mice inoculated with ETEC cells incubated with serum harvested from mice submitted to the prime-boost immunization regimen were protected with survival rates of 80%, 95% and 100% with serum pools collected from mice boosted with LDV6 cells, LDVanc3 cells and LDV6 spores, respectively (Table 1). Curiously, no protective effect was detected with serum samples collected from mice spores of

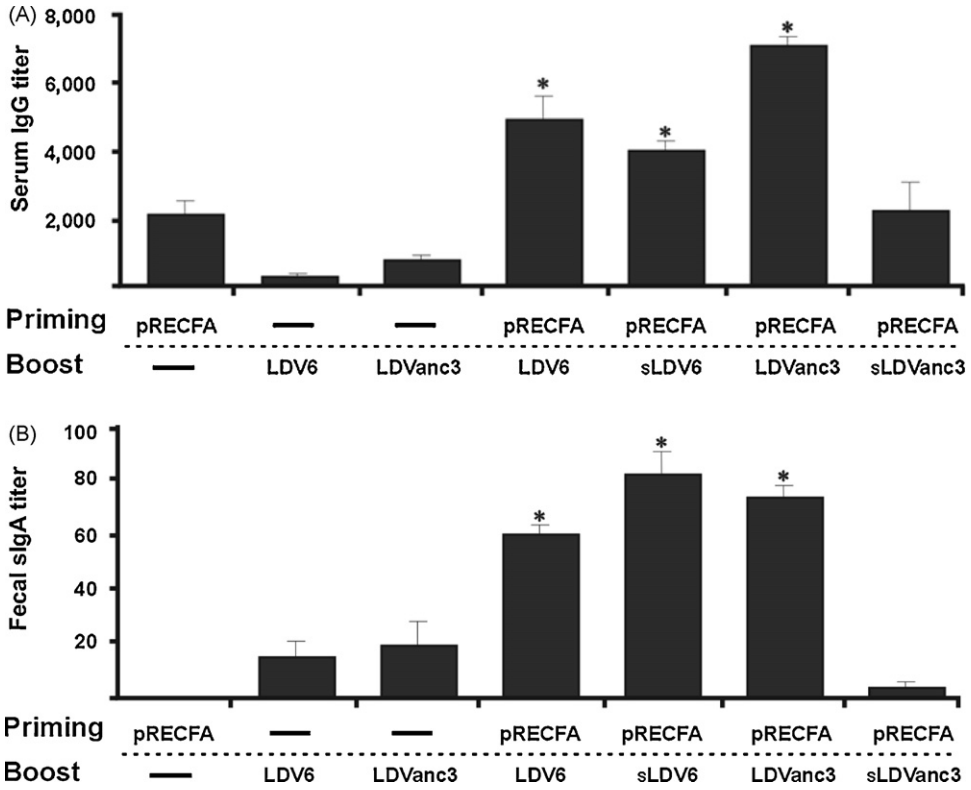

Fig. 2. CfaB-specific antibody responses elicited in female DBA/2 mice submitted to the prime/boost immunization regimen. Serum IgG (A) and fecal IgA (B) responses (means ± S.E.) were measured in pooled serum samples collected from mouse groups immunized with two i.m. doses (100 μg/each) of pRECFA (pRECFA), two p.o. doses (three consecutive daily doses containing 10^{10} CFU) of vegetative cells of the recombinant *B. subtilis* LDV6 (LDV6) or LDVanc3 (LDVanc3) strains, or the prime/boost immunization regimen in which vegetative cells (pRECFA + LDV6 or LDVanc3) or spores (pRECFA + LDV6s or LDVanc3s) were administered to mice previously primed with 2 pRECFA doses. Statistically significant differences ($p < 0.05$) with regard to the pRECFA-immunized group (A) or mice immunized with the *B. subtilis* LDV6 strain are indicated by asterisks.

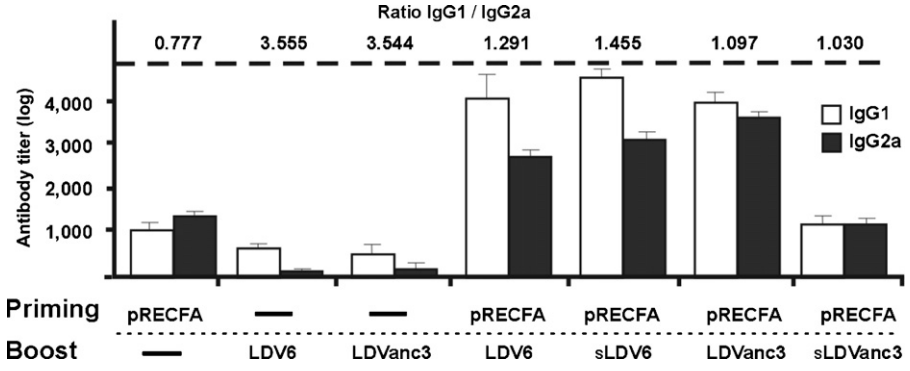

Fig. 3. Serum anti-CfaB IgG subclass responses elicited in mice subjected to the DNA priming/*B. subtilis* boosting immunization regimen. Mouse groups were immunized with the same immunization regimens described in the legend of Fig. 2. Values are expressed as means of serum pool IgG1 and IgG2 titers (±S.E) of each immunization group. The IgG1/IgG2a ratios are indicated on the top of the figure.

the LDVanc3 strain. Mice immunized with two doses of vegetative cells of the *B. subtilis* strains LDV6 and LDVanc3 generated serum samples conferring protection levels of 23.5% while no protection was observed with serum samples harvested from mice vaccinated with pRECFA DNA vaccine (Table 1). These results clearly demonstrate that antibodies raised in mice subjected to the combined DNA/*B. subtilis* vaccine regimen bind and neutralize the adhesive properties of the CFA/I fimbriae conferring protection to challenge with a wild type CFA/I^+ ETEC strains.

As final demonstration of the protective potential of the tested priming/boosting vaccine regimen, we performed lethal challenges with the CFA/I^+ ETEC strain using neonate mice to evaluate the passive protection conferred by vaccinated dams secreting milk anti-CfaB IgA antibodies, as previously demonstrated [15]. As indicated in Fig. 4, DBA/2 dams submitted to the prime/boost vaccine regimen employing cells of the LDVanc3 and spores of the LDV6 strain conferred full protection to newborns challenged with the ETEC 258909-3 strain. Similarly, dams boosted with vegetative cells of the LDV6 strain conferred 87% (7 survivors among 8 challenged pups) protection to their offsprings, while no protection was observed among litters fostered by dams boosted with spores of the LDVanc3 strain. Protection levels measured in mice vaccinated with two doses of the vegetative cells of both *B. subtilis* LDV6 and LDVanc3 strains achieved 20% and 22% of the inoculated neonates, respectively. As previously shown [15,16], dams vaccinated with pRECFA conferred no passive protection to ETEC

Table 1
Inhibition of CFA/I-mediated haemagglutination by CfaB-specific antibodies raised in mice submitted to the DNA priming/*B. subtilis* boosting vaccine regimen

Vaccine regimen[a]	IgG-ELISA titer ± S.E.[b]	IHA titer[c]	Neutralization of in vitro lethality[d]
pRECFA	2,139 ± 245	0	0/19 (0)
LDV6	319 ± 121	2	4/17 (24.5)
LDVanc3	757 ± 92	2	4/17 (24.5)
pRECFA + LDV6	4,897 ± 324	1500	12/15 (80)
pRECFA + sLDV6	4,002 ± 298	1300	18/18 (100)
pRECFA + LDVanc3	7,077 ± 424	2500	18/19 (94.8)
pRECFA + sLDVanc3	2,890 ± 122	0	0/17 (0)
Nonimmunized	ND	0	0/20 (0)

[a] DBA/2 mice were immunized with 2 i.m. doses of pRECFA (100 μg/each dose), or two oral doses of *B. subtilis* LDV6 or LDVanc3 strains, either spores (sLDV6 and sLDVanc3) or vegetative cells (LDV6 and LDVanc3) (each dose consisting of three consecutive daily dose of 10^{10} cells or spore), or the priming/boosting regimen based on two i.m. dose of pRECFA followed two weeks later by two oral doses with the recombinant *B. subtilis* strains; The monoclonal antibody Mab-84 was used as positive control.

[b] Anti-CFA/I IgG ELISA titers of serum pools harvested from mice submitted to the different immunization regimens. Values expressed as means ± S.E.

[c] Maximal serum dilutions giving positive results in IHA tests.

[d] Survival rates of DBA/2 neonate mice challenged with the ETEC 258909 strain (2×10^7 CFU) previously incubated for 1 h with the tested serum samples. Values are expressed as number of surviving animals/total number of inoculated mice. Numbers in parenthesis represented percentage of survivors. All tested serum samples were diluted 1:25 before incubation with the ETEC cells.

challenges. These results further demonstrate that vaccination of mice with the prime/boost immunization regimen, based on *B. subtilis* vaccine strains, confers protective passive immunity to CFA/I$^+$ ETEC-associated lethality in newborn DBA/2 mice.

4. Discussion

Development of vaccine vehicles based on genetically modified *B. subtilis* strains has received growing interest since the demonstration that strains expressing different target antigens, either on the surface of spores or produced by vegetative cells, can elicit both systemic and secreted antibody, as well as cellular immune responses, to the passenger antigens following oral or parenteral administration to mice [1–4,8,10]. In the present study, we further investigated the potential use of *B. subtilis*-based vaccine vehicles studying the ability of recombinant *B. subtilis* to boost secreted and systemic specific antibody responses in mice previously primed with a DNA vaccine encoding the structural subunit of the CFA/I frimbriae produced by human-derived ETEC strains. Our results clearly show that orally delivered recombinant spores or vegetative cells synergistically enhanced the anti-CfaB specific antibody responses but, most importantly, increase the affinity of the generated antibodies to the native CFA/I fimbriae expressed by live ETEC cells, such as demonstrated by the inhibition of binding properties of fimbriated bacteria and the complete passive protection conferred by vaccinated dams to newborn DBA/2 mice challenged with a virulent CFA/I$^+$ ETEC strain. Collectively, these results reinforce the vaccine potential of recombinant *B. subtilis* strains as an alternative vaccine vehicle and represent an additional tool for the development of vaccines against enteric non-invasive pathogens as well as other pathogens.

Repeated oral exposure of recombinant *B. subtilis* vaccine strains, a procedure required to circumvent the non-colonizing nature of this bacterial species, has been shown to limit further increments in systemic and secreted antibody responses elicited in vaccinated mice [10]. As shown in the present study, boosting the immune system of mice previously primed with a DNA vaccine encoding the same antigen reduced the number of *B. subtilis* vaccine doses required to achieve specific antibody levels otherwise not obtained with the DNA vaccine or the *B. subtilis* strain in multiple dose vaccine regimens. Additionally, serum anti-CfaB IgG responses reached in mice boosted with the *B. subtilis* strains were similar to those obtained with an attenuated *Salmonella* Typhimurium strain, which retains ability to transiently colonize and multiply in the intestinal epithelia and subjacent tissues [15,16]. Therefore, our results indicate that recombinant *B. subtilis* strains may replace attenuated pathogens, such as *Salmonella* and *Mycobacteria*, as oral delivered vehicle in prime-boost immunization regimens without the inherent virulence reversion risk but preserving similar immune

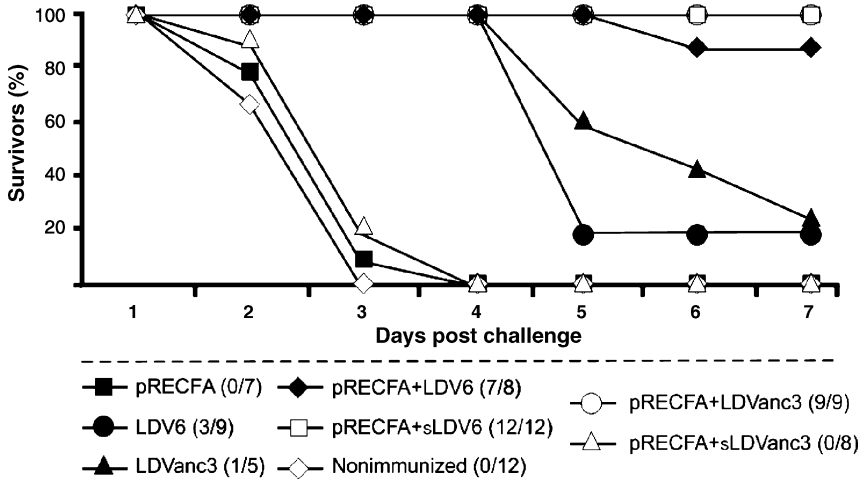

Fig. 4. Survival curves of DBA/2 newborn mice challenged with lethal loads of a virulent ETEC strain and nursing from dams submitted to different vaccine regimens. Female DBA/2 mice were submitted to immunization regimens described in the legend of Fig. 2 and indicated in the figure inset.

stimulation potency, as evaluated by the induced antigen-specific antibody responses.

Heterologous prime-boost vaccine regimen, in which animals are primed with one vector and boosted with the same antigen delivered by a second vector in a different context, has usually been tested with DNA vaccines as the priming stimulus and recombinant poxviruses or adenoviruses as the boosting components for mounting synergic humoral and cellular-based specific immune responses [24–26]. So far, only a few reports described the use of live bacterial strains, such as attenuated *Salmonella enterica* vaccine strains, as the priming or boosting vector for parenteral or mucosal delivery of vaccine antigens [27,28]. Based on our own experience, attenuated *S.* Typhimurium strains have shown a good performance as orally delivered vaccine vehicles for boosting systemic and secreted specific antibody responses in mice primed with a DNA vaccine encoding the CfaB antigen [14,15]. Since similar enhancement of the elicited CfaB-specific antibody responses could be achieved in mice immunized with recombinant *B. subtilis*, a bacterial species granted with the GRAS status, the natural concern associated with the administration of attenuated bacterial host is eliminated both for human and animal use. The present evidences indicate that recombinant *B. subtilis* strains, either as vegetative cells or spores, may replace with obvious advantages attenuated pathogens, such as *Salmonella* and mycobacteria, in prime-boost immunization regimens.

The vaccine potential of recombinant *B. subtilis* strains has also been demonstrated as a priming vehicle in a prime-boost vaccination regimen [23]. For such purpose, orally delivered recombinant spores expressing the tetanus toxin fragment C (TTFC) have been shown to efficiently prime immune responses in mice parenterally boosted with the purified protein for TTFC-specific antibody responses [23]. Our data further support and extend the analyses of the potential use of recombinant *B. subtilis* vaccine vehicles and demonstrated that specific antibody responses are also boosted by vaccine strains, either as spores or vegetative cells, in animals previously primed with an antigen coded by DNA vaccines.

The lack of a good ETEC animal model has restricted efficacy studies of potential vaccines prior to their administration to volunteers, which are presently feasible at only a few places in the world. As an attempt to evaluate the protective efficacy of the proposed ETEC vaccine regimen, we adapted a previously described [16] murine neonate challenge model to an ETEC strain isolated from a human host. Based on that model it was possible to demonstrate that the serum antibodies raised in mice submitted to the DNA priming/*Salmonella* boosting vaccine regimen confer almost complete protection to lethal challenges with a CFA/I^+ ETEC strain. As expected, sera from mice immunized only with the DNA vaccine did not confer any protection to the inoculated mice, a reflection of the preferential targeting of the induced antibodies to linear epitopes not exposed on the surface of intact CFA/I fimbriae [20]. Such observations further indicate that antibodies raised in mice submitted to the combined vaccine regimen recognize epitopes required for the binding of ETEC to host cell receptors. Nonetheless, definite demonstration of the protective potential of the proposed vaccine regimen will require further testing in other animal model, as the removable intestinal tie-adult rabbit diarrhea model, or human volunteers.

So far vaccines against ETEC-associated diarrhea have been mainly restricted to mixtures of inactivated ETEC strains producing different colonization factors or attenuated *Salmonella* or *Shigella* strains genetically modified to express ETEC adhesins [12,29]. The present results indicated that the vaccine regimen based on priming with a DNA vaccine followed by boosting with a recombinant *Salmonella* strain, expressing the same ETEC fimbrial protein, can be a paradigm for a new generation of vaccines for enteric pathogens based on the prime-boost concept, which has been successfully used against several virus- or parasite-associated diseases. Constructions based on multi-epitope DNA vaccines or combination of several plasmids encoding different ETEC adhesions are feasible and may help to develop multi-target ETEC as well as other enteric pathogens with heterogenous antigen composition.

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