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Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner

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Abstract

We report the use of CotC, a major component of the *Bacillus subtilis* spore coat, as a fusion partner for the expression of two heterologous antigens on the spore coat. Recombinant spores expressing tetanus toxin fragment C (TTFC) of *Clostridium tetani* or the B subunit of the heat-labile toxin of *Escherichia coli* (LTB) were used for oral dosing and shown to generate specific systemic and mucosal immune responses in a murine model. This report, expanding the previously described expression of TTFC on the spore surface by fusion to CotB [J Bacteriol 183 (2001) 6294] and its use for oral vaccination [Infect Immun 71 (2003) 2810] shows that different antigens can be successfully presented on the spore coat and supports the use of the spore as an efficient vehicle for mucosal immunisation.

Keywords: Vaccines; Spores; *B. subtilis*

1. Introduction

Strategies to control and eradicate emerging and re-emerging pathogens are often either not available or subject to important limitations, thus prompting many studies for the development of new, more effective and safer vaccination strategies. In particular, considerable efforts have recently been devoted to the development of oral vaccines, that are able to provide better levels of local immunity against pathogens which enter the body primarily through the mucosal surface [3]. Since mucosal immunisation using soluble antigens has long been known to generate poor immune responses due to antigen degradation in the stomach, limited absorption and tolerance, different approaches have been undertaken to develop carrier systems displaying heterologous antigens on the surface of microbial cells and viruses.

Delivery systems so far developed to improve the mucosal immune responses fall into two general categories, non-living and living. Non-living systems include liposomes, microparticles, immune stimulating complexes (ISCOMS), and formulations based on cholera toxin and *Escherichia coli* LT toxins [4,5]. Live carrier systems include both plants and bacteria [3,6]. The bacterial systems for heterologous antigen presentation have attracted considerable interest but because these rely largely on live attenuated pathogens such as *Salmonella* and Mycobacteria considerable safety concerns remain.

The Gram positive bacterium *Bacillus subtilis* has been extensively studied as a model prokaryotic system with which to understand gene regulation and the transcriptional control of unicellular differentiation [7]. This organism is regarded as a non-pathogen and is classified as a novel food which is currently being used as a probiotic for both human and animal consumption [8]. The distinguishing feature of this micro-organism is that it produces an endospore as part of its developmental life cycle when starved of nutrients. The mature spore, when released from its mother cell can survive in a metabolically dormant form indefinitely. The spore offers unique resistance properties and can survive extremes of temperature, dessication and exposure to solvents and other noxious chemicals [9]. These unique attributes would make the spore an attractive vehicle for delivery of heterologous antigens or, indeed, any bioactive molecule, to extreme environments such as the gastrointestinal tract. We have recently reported the development of a surface display system based on the use of CotB [1], a protein component of the spore coat, as a fusion partner to express a highly immunogenic tetanus toxin fragment C (TTFC) on

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the spore surface. We have also shown that when administered orally spores expressing the CotB-TTFC chimera on their surfaces can protect mice from an otherwise lethal challenge of tetanus toxin [2]. This seminal and important finding shows the potential of using recombinant spores as heat stable, oral, vaccine vehicles.

Here, we have expanded our previous findings showing that it is possible to use CotC, another protein component of the *B. subtilis* spore coat [10], as fusion partner for the expression of two heterologous antigens. In this study, we used two model antigens, tetanus toxin fragment C from *Clostridium tetani* [11] and the B subunit of the heat-labile toxin of *E. coli* (LTB) [5]. Both antigens have been used extensively to evaluate bacteria as vaccine delivery vehicles [11–15]. Induction of local and systemic immune responses after oral administration of recombinant spores expressing CotC-TTFC or CotC-LTB chimeras points to the spore, and specifically the spore coat, as a novel and potentially powerful system to display heterologous antigens.

2. Materials and methods

2.1. Bacterial strains and transformation

B. subtilis wild type strain PY79 (*spo*$^+$; [16]) was used. All recombinant strains described here are isogenic derivatives of PY79. Plasmid amplification for nucleotide sequencing, subcloning experiments and transformation of *E. coli* competent cells were performed in the *E. coli* strain DH5α [17]. Bacterial strains were transformed according to previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells [17] and two-step transformation of *B. subtilis* [18].

2.2. Construction of gene fusions

Construction of gene fusions: In order to obtain both *cotC*-based gene fusions *cotC* DNA was first amplified by PCR using the *B. subtilis* chromosome as template and CotCp and CotCa oligonucleotides (acatgcatgcTGTAGGATAAATCGTTTG and gaaagatctGTAGTGTTTTTTATGCTT, respectively, annealing at −179/−162 and +180/+197 of *cotC*; capital and small letters indicate bases of complementarity with *cotC* and an unpaired tail carrying a restriction site) as primers. An amplification product of the expected size (395 bp) was cloned into the pGem-Teasy vector (Promega) yielding plasmid pGEM-CotC. To construct the *cotC*: :*tetC* gene fusion plasmid, pGEM-CotC was sequentially digested with *Sph*I and *Bgl*II to release *cotC* DNA and the 395 bp fragment cloned in frame to the 5′ end of the *tetC* gene carried by plasmid pGEM-TTFC [19], yielding plasmids pRH21. To obtain the *cotC*: :*eltB* gene fusion, a 333 bp DNA fragment coding for LTB was PCR amplified using plasmid pSMB120 [15] as a template and oligonucleotides LTB1 and LTB2 (tcatccagatctttcGCTCCTCAGTCTATTAC and gcgtcgacAGTTTTCCATACTGATTGC, respectively, annealing at +64/+90 and +355/+373 of *eltB*; capital and small letters indicate bases of complementarity with *eltB* and an unpaired tail carrying a restriction site) as primers. The PCR product was cloned into the pGEM-Teasy yielding plasmid pGEM-T-LTB. Plasmid pGEM-T-LTB was sequentially digested with *Bgl*II and *Sal*I and cloned in frame to the 3′ end of the *cotC* gene carried by plasmid pGEM-CotC, yielding plasmid pM10.

Plasmids pRH21 and pM10 were digested with *Sph*I and *Sal*I, the fragments carrying the gene fusions were gel-purified and ligated into plasmid pDG364 [18] previously digested with the same two restriction enzymes. *E. coli* competent cells were transformed with the ligation mixture and the selected ampicillin-resistant clones screened by restriction analysis of their plasmids. Individual clones for each transformation were selected, named pRH22 and pIM51 (from pRH21 and pIM10, respectively), and used to determine the nucleotide sequence of the inserted DNA.

2.3. Chromosomal integration

Plasmids pRH22 and pIM51 were linearized by digestion with enzyme *Pst*I and/or *Pvu*II and used to transform competent cells of the *B. subtilis* strain PY79. Chloramphenicol-resistant (Cm^R) clones were the result of a double cross-over recombination, resulting in the interruption of the non-essential *amyE* gene on the *B. subtilis* chromosome (Fig. 1C). Several Cm^R clones were tested by PCR using chromosomal DNA as a template and oligonucleotides AmyS and AmyA [1] to prime DNA amplification. Clones deriving from plasmids pRH22 showed an amplification product of 3345 bp, while clones deriving from plasmid pIM51 showed a smaller amplification product (2265 bp), thus indicating the occurrence of correct recombination events. Two clones, one for each transformation, were named RH114 (from pRH22, Fusion A in Fig. 1) and IM201 (from pIM22, Fusion B in Fig. 1) and kept for further studies. Both fusions were moved into a *cotC* null mutant strain by chromosomal DNA-mediated transformation [18]. Chromosomal DNA extracted from strains RH114 and IM201 was used to transform the isogenic *cotC* null strain RH101. RH101 was obtained by transforming strain DL071 [10], with plasmid pJL62 carrying the *cat* gene interrupted by the *spc* gene (spectinomycin resistance, Sp^R). Sp^R clones were the result of a double recombination event interrupting *cat*. Several Sp^R clones were selected and one of them, RH101, used for further studies.

2.4. Preparation of spores

Sporulation of either PY79, RH114 (CotC-TTFC) or IM201 (CotC-LTB) was made in DSM (Difco-sporulation media) using the exhaustion method as described elsewhere [20]. Sporulating cultures were harvested 24 h after the initiation of sporulation. Purified suspensions of spores

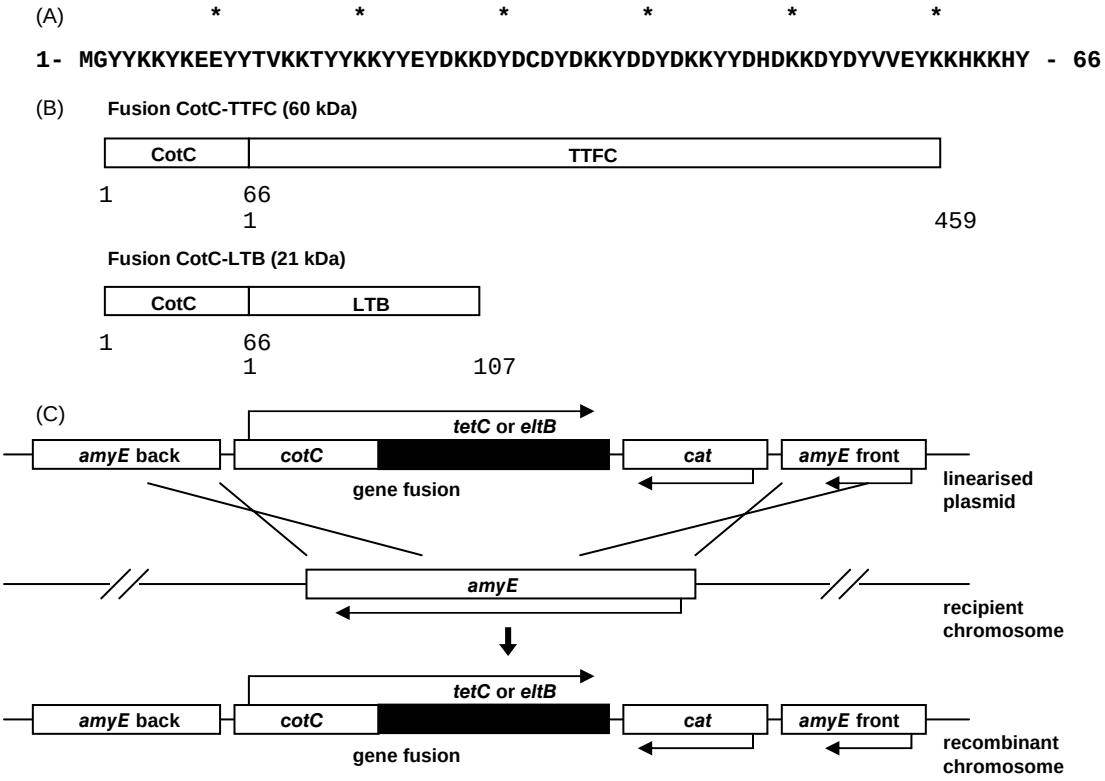

Fig. 1. Cloning strategy. (A) CotC amino acid sequence [10]. (B) Schematic representation of the two fusion proteins constructed. (C) Strategy for the chromosomal integration of the two gene fusions. Arrows indicate direction of transcription.

were made as described by Nicholson and Setlow [20] using lysozyme treatment to break any residual sporangial cells followed by washing in 1 M NaCl, 1M KCl and water (two-times). PMSF (0.05 M) was included to inhibit proteolysis. After the final suspension in water spores were treated at 65 °C for 1 h to kill any residual cells. Next, the spore suspension was titred immediately for CFU/ml before freezing at −20 °C. Using this method, we could reliably produce 6×10^{10} spores per litre of DSM culture. Each batch of spores prepared in this way was checked for the presence of the 60 kDa CotC-TTFC protein or the 21 kDa CotC-LTB hybrid proteins in extracts of spore coat protein by Western blotting using a polyclonal TTFC or LTB antiserum.

2.5. Extraction of spore coat proteins

Spore coat proteins were extracted from suspensions of spores at high density (>1×10^{10} spores per ml) using an SDS-DTT extraction buffer as described in detail elsewhere [20]. Extracted proteins were assessed for integrity by SDS-polyacrylamide gel (PAGE) and for concentration by two independent methods: the Pierce BCA Protein Assay (Pierce) and the BioRad DC Protein Assay kit (Bio-Rad).

2.6. Western and dot-blot analysis

Western blot filters were visualised by the ECL (Amersham Pharmacia Biotech) method following the manufacturer's instruction. Serial dilutions of extracted proteins and of purified TTFC or LTB were used for dot-blot analysis. Filters were then visualised by the BCIP/NBT Color Development Solution (Bio-Rad) or by the ECL (Amersham Pharmacia Biotech) method and subjected to densitometric analysis by Fluor-S Multimager (Bio-Rad). CotC-, TTFC-and LTB-specific antibodies were raised against 15 amino acid synthetic peptides designed on the base of the C-terminal region of the respective proteins (IGtech, Salerno, Italy).

2.7. Indirect ELISA for detection of antigen-specific serum and mucosal antibodies

Plates were coated with 50 μl per well of the specific antigen (2 μg/ml in carbonate/bicarbonate buffer) and left at room temperature overnight. Antigen was either TTFC or LTB purified protein. After blocking with 0.5% BSA in PBS for 1 h at 37 °C serum samples were applied using a two-fold dilution series starting with a 1/40 dilution in ELISA diluent buffer (0.1 M Tris–HCl, pH 7.4; 3% (w/v) NaCl; 0.5% (w/v) BSA; 10% (v/v) sheep serum (Sigma); 0.1% (v/v) Triton-X-100; 0.05% (v/v) Tween-20). Every plate carried replicate wells of a negative control (a 1/40 diluted pre-immune serum), a positive control (serum from mice immunised parentally with either TTFC or LTB purified protein). Plates were incubated for 2 h at 37 °C before addition of anti-mouse HRP conjugates (Sigma). Plates were

incubated for a further 1 h at 37 °C then developed using the substrate TMB (3,3′,5,5′-tetramethyl-benzidine; Sigma). Reactions were stopped using 2 M H_2SO_4. Dilution curves were drawn for each sample and end-point titres calculated as the dilution producing the same optical density as the 1/40 dilution of a pooled pre-immune serum. Statistical comparisons between groups were made by the Mann–Whitney *U*-test. A *P*-value of >0.05 was considered non-significant. For ELISA analysis of faecal IgA, we followed the procedure of Robinson et al. [21] using approximately 0.1 g faecal pellets that had been suspended in PBS with BSA (1%) and PMSF (1 mM), incubated at 4 °C overnight and then stored at −20 °C prior to ELISA. For each sample, the end-point titre was calculated as the dilution producing the same optical density as the undiluted pre-immune faecal extract.

2.8. Immunisations

Groups of eight mice (female, C57 BL/6, 8 weeks) were immunised by either the intra-peritoneal or oral route with suspensions of either spores expressing CotC-TTFC (RH114), CotC-LTB (IM201) or control, non-expressing, spores (strain PY79). For oral dosings mice were lightly anaesthetised with halothane using a regime based on previous work optimising mucosal immunisations [21,22]. A naïve, non-immunised control group was included. Intra-peritoneal injections contained 1.5×10^9 spores in a volume of 0.1 ml administered on days 0, 14 and 28. Serum samples were taken on days −1, 13, 27, 43 and 82. Oral immunisations contained 1.0×10^{10} spores in a volume of 0.15 ml and were administered by intra-gastric lavage on days 0–2, 16–18, 33–35. Serum samples were collected on days −1, 15, 32 and 68 and faeces on days −1, 15, 32 and 53.

2.9. Toxin binding inhibition (ToBI) test for measurement of neutralising antibody levels

ELISA plates (Immuno Maxisorp, Nunc) were coated with 80 μl per well of horse tetanus antitoxin (1 IU/ml in carbonate/bicarbonate buffer) and incubated at 37 °C overnight. Neutralising reaction was carried out using another set of plates that had been blocked with 1% BSA in PBS at 37 °C for 1 h. In the reaction, serum samples were applied using a two-fold dilution series, mixed with equal volumes of tetanus toxoid (0.005 IU/ml in PBS, R.I.V.M., The Netherlands) and then incubated at 37 °C overnight. Dilutions starting with 1 IU/ml of a tetanus antitoxin reference (WHO) were also included. After the reaction, corresponding wells were transferred from neutralising plates to ELISA plates which were then incubated for 2 h at 37 °C. Remaining tetanus toxoid after neutralisation was detected by further incubation of plates with tetanus antitoxin peroxidase conjugate (R.I.V.M., The Netherlands) for 1 h at 37 °C, and development using TMB substrate (Sigma). The levels of tetanus antitoxin in serum samples were calculated from the standard curve drawn with tetanus antitoxin reference data.

3. Results

3.1. CotC as a fusion partner

At least 25 polypeptides are organised to form the *B. subtilis* spore coat [23]. Some of these polypeptides have been associated with the outer layer of the coat but only one of them, CotB, has so far been localised on the spore surface and, based on this surface display, employed as a fusion partner to express the tetanus toxin fragment C on the spore surface [1,2]. In order to identify additional routes for expressing heterologous antigens on the spore surface, we searched for other components of the spore coat to be used as fusion partner. Based on its association with the outer layer of the spore coat and on its relative abundance within the coat structure we focused our attention on CotC (Fig. 1A), a 8.8 kDa component of the outer coat, rich in tyrosine (30.3%), lysine (28.8%) and aspartic acid (18.2%) residues [10].

As model antigens, we used: (i) the 459 amino acid C-terminal fragment of the tetanus toxin, a well characterised and highly immunogenic [24] 51.8 kDa peptide, encoded by the *tetC* gene of *C. tetani*; and (ii) the 103 amino acid B subunit of the heat-labile toxin of *E. coli*, a 12 kDa peptide, encoded by the *eltB* gene [5] (Fig. 1B). The strategy to obtain recombinant *B. subtilis* spores expressing TTFC or LTB on their surface was based on (i) use of the *cotC* gene and of its promoter for the construction of translational fusions and on (ii) chromosomal integration of the *cotC-tetC* or *cotC-etlB* gene fusions into the coding sequence of the non-essential gene *amyE* [18]. Both CotC-TTFC and CotC-LTB gene fusions were obtained by cloning *tetC* or *eltB* in frame with the 3′ codon of *cotC* (Fig. 1B) in the integrative vector pDG364 [18]. Both fusions were integrated into the *B. subtilis* chromosome at the *amyE* locus by double cross-over recombination events (Fig. 1C; Section 2). Individual clones for each transformation were tested by PCR (not shown), named RH114 (CotC-TTFC) and IM201 (CotC-LTB) and used for further analysis. The two recombinant strains and their isogenic parental strain PY79 showed comparable sporulation and germination efficiencies and their spores were equally resistant to chloroform and lysozyme treatment (not shown), indicating that the presence of CotC-TTFC or CotC-LTB fusion did not significantly affect spore structure and/or function.

3.2. Expression of TTFC or LTB in the spore coat

Western blot analysis of the coat protein fraction purified from spores of wild type and recombinant strains revealed the presence of an approximately 60 kDa polypeptide which reacted with both TTFC- and CotC-specific antibodies and

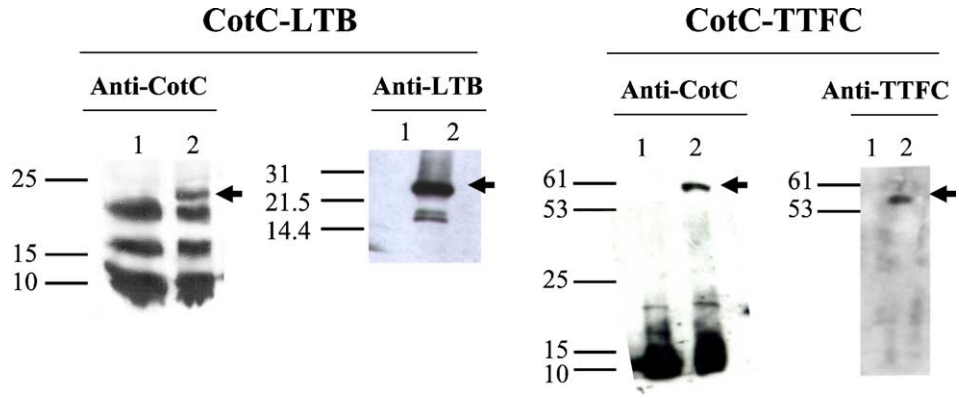

Fig. 2. Western blot of proteins extracted from purified spores of strains IM201 (CotC-LTB) and RH114 (CotC-TTFC) and reacted against anti-CotC, anti-LTB and anti-TTFC antibodies. Fifteen micrograms of total proteins from strains PY79 (lanes 1 in all panels), IM201 (lanes 2, Panel CotC-LTB) and RH114 (lanes 2 Panel CotC-TTFC) were fractionated on 10% polyacrylamide gel and upon electro-transfer on nitrocellulose membranes, reacted with primary anti-rabbit antibodies, then with secondary antibodies and visualised as described in Section 2. Arrows point to fusion proteins. Molecular weight markers (kDa) are indicated.

a 21 kDa polypeptide which reacted with both LTB- and CotC-specific antibodies (Fig. 2). Additional polypeptides of 12 and 21 kDa, specifically reacting with CotC-specific antibodies, were present in extracts from wild type and recombinant spores (Fig. 2). These additional polypeptides are due to the ability of CotC to assemble in multiple forms in the spore coat [25].

CotC and the two chimeric proteins were not found by western blot analysis with anti-CotC antibodies of the coat protein fraction extracted from spores of strains PY79, RH114 and IM201 in which the *cotE* gene had been deleted (data not shown). Since *cotE* is known to encode a morphogenetic protein required for outer coat assembly [26], this CotE requirement suggests that CotC-containing chimeras are assembled within the outer spore coat. In order to analyse whether an intact copy of CotC was needed for surface expression of CotC-based fusions we moved, by chromosomal DNA-mediated transformation, the gene fusions into a *cotC* mutant strain (RH101). Western blot experiments, performed with coat proteins from strains carrying the gene fusions in the absence of an intact copy of *cotC* showed the presence of CotC-TTFC and CotC-LTB specific polypeptides (Fig. 3). However, in the presence of an intact copy of *cotC* both fusion proteins were expressed with higher efficiency (Fig. 3), thereby indicating that the *cotC* gene product is needed to obtain optimal expression of the CotC-based fusion proteins within the spore coat structure. Based on this result, strains RH114 and IM201 carrying the CotC-based chimera in the presence of an intact copy of *cotC* were selected for further analysis. We also verified that TTFC and LTB were surface exposed by immunofluorescence. Using polyclonal sera against TTFC and LTB we could detect both antigens in sporulating cultures harvested at 6 h following the initiation of spore formation but this labelling did not occur in the isogenic non-recombinant strain PY79 (data not shown).

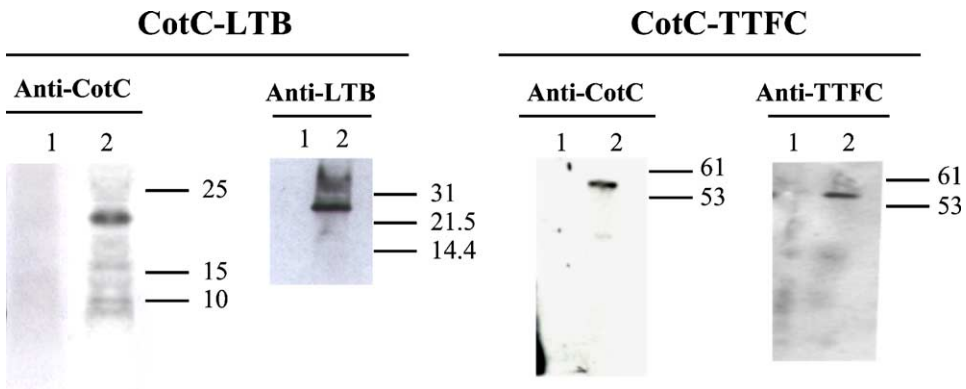

Fig. 3. Western blot of proteins extracted from purified spores of strains carrying the CotC-LTB or CotC-TTFC fusion but deleted of the *cotC* gene. Fifteen micrograms of total proteins from strains PY79 (lanes 1 in all panels), IM201 (lanes 2, Panel CotC-LTB) and RH114 (lanes 2 Panel CotC-TTFC) were fractionated on 10% polyacrylamide gel and upon electro-transfer to nitrocellulose membranes, reacted with CotC-specific rabbit antibodies, then with secondary antibodies and visualised as described in Section 2. Molecular weight markers (kDa) are indicated.

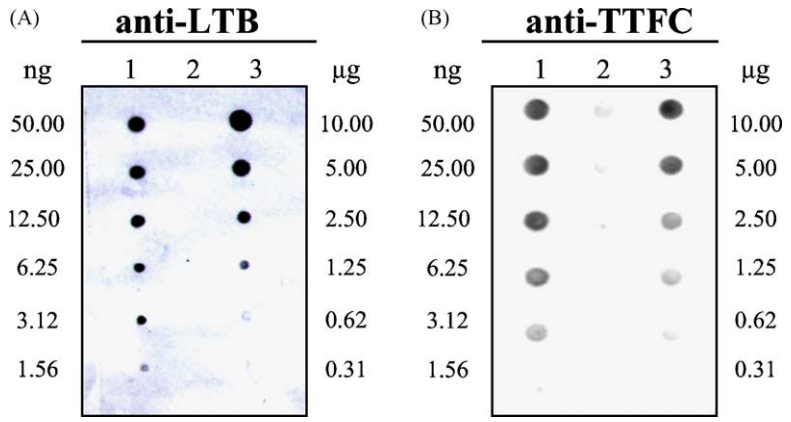

Fig. 4. Quantification of expressed antigen. Dot blot experiments performed with the indicated concentrations of coat proteins (in μg) extracted from spores carrying the CotC-TTFC or CotC-LTB fusion in otherwise wild type background (lanes 3) and from wild type spores (lanes 2). Purified TTFC or LTB (in ng, lanes 1) were also utilised. Anti-TTFC and anti-LTB primary antibodies and secondary anti-rabbit peroxidase-conjugated antibodies were used. Reactions were visualized by NCP/BCIP or ECL as described in Section 2.

3.3. Efficiency of TTFC and LTB presentation

A quantitative determination of the amount of TTFC or LTB exposed on *B. subtilis* spores was obtained by dot blot experiments using serial dilutions of purified TTFC or LTB and of coat proteins extracted from spores of the wild type and the recombinant strains. Proteins were reacted with anti-TTFC and anti-LTB antibody, then with alkaline phosphatase-conjugated secondary antibodies and colour developed by the BCIP/NBT or ECL system (Bio-Rad). Fig. 4 shows the results obtained with strains RH114 and IM201, carrying fusion CotC-TTFC or CotC-LTB, respectively. A densitometric analysis indicated that both fusion proteins amounted to 0.3% of total coat proteins extracted. Since in our experimental conditions, an average of 0.032 (±2%) pg of total coat proteins was reproducibly extracted from each spore of the recombinant strains by SDS-DTT treatment at 65 °C (see Section 2), we calculated that 9.6×10^{-5} pg of CotC-TTFC or CotC-LTB fusion protein was extracted from each spore. Based on that and on the deduced molecular weight of 60 and 21 kDa for the two recombinant proteins, we estimated that ca. 9.7×10^2 and 2.7×10^3 molecules of CotC-TTFC and CotC-LTB, respectively, were extracted from each spore.

3.4. Serum anti-TTFC and anti-LTB responses following intra-peritoneal injection of recombinant spores expressing TTFC or LTB

Immunogencity of recombinant spores was determined by intra-peritoneal injection of groups of eight C57 mice (Fig. 5). Our immunisation schedule used three injections (containing 1.5×10^9 spores per dose) of either recombinant RH114 spores (expressing hybrid CotC-TTFC), recombinant IM201 spores (expressing hybrid CotC-LTB) or non-recombinant PY79 spores. Since each recombinant spore was shown to carry approximately 9.6×10^{-5} pg of CotC-LTB or CotC-TTFC, our immunising dose would contain 0.14 μg of CotC-LTB or CotC-TTFC fusion protein, which would correspond to 0.12 μg of TTFC or 0.08 μg of LTB per injection. Immunisation with RH114 spores resulted in peak anti-TTFC IgG titres ($\sim 5 \times 10^2$) at day 43 (Fig. 5A). Similarly, immunisation with IM201 spores resulted in peak anti-LTB titres ($\sim 2 \times 10^2$) at day 43 (Fig. 5B) although these levels were somewhat lower than that observed with CotC-TTFC but significantly different from control groups ($P < 0.05$). This demonstrated that both TTFC and LTB were stably expressed and appropriately immunogenic when displayed on the spore surface.

3.5. Serum anti-TTFC and anti-LTB responses following oral immunisation

Groups of eight mice were dosed orally with 1×10^{10} spores per dose (0.96 μg of CotC-TTFC or CotC-LTB, i.e. 0.82 μg TTFC or 0.55 μg LTB per oral dose). As shown in Fig. 6A oral immunisation of mice with RH114 (CotC-TTFC) spores gave anti-TTFC IgG titres greater than 2×10^2 on day 68. These were found to be significantly above ($P < 0.05$) those of mice dosed with non-recombinant spores (PY79) or the control naïve group. Oral immunisation of mice with IM201 (CotC-LTB) spores though only produced an anti-LTB serum IgG response, not significantly above ($P > 0.05$) those of mice dosed with non-recombinant spores (PY79) (Fig. 6B).

3.6. Mucosal anti-TTFC and anti-LTB IgA responses

Fresh faecal pellets from mice immunised orally with RH114 or IM201 spores were tested for the presence of TTFC-specific or LTB-specific secretory IgA (sIgA) by ELISA. Immunisation with spores expressing CotC-TTFC or CotC-LTB elicited, respectively, clear TTFC- (Fig. 6C) or LTB-specific (Fig. 6D) sIgA responses. In the case of

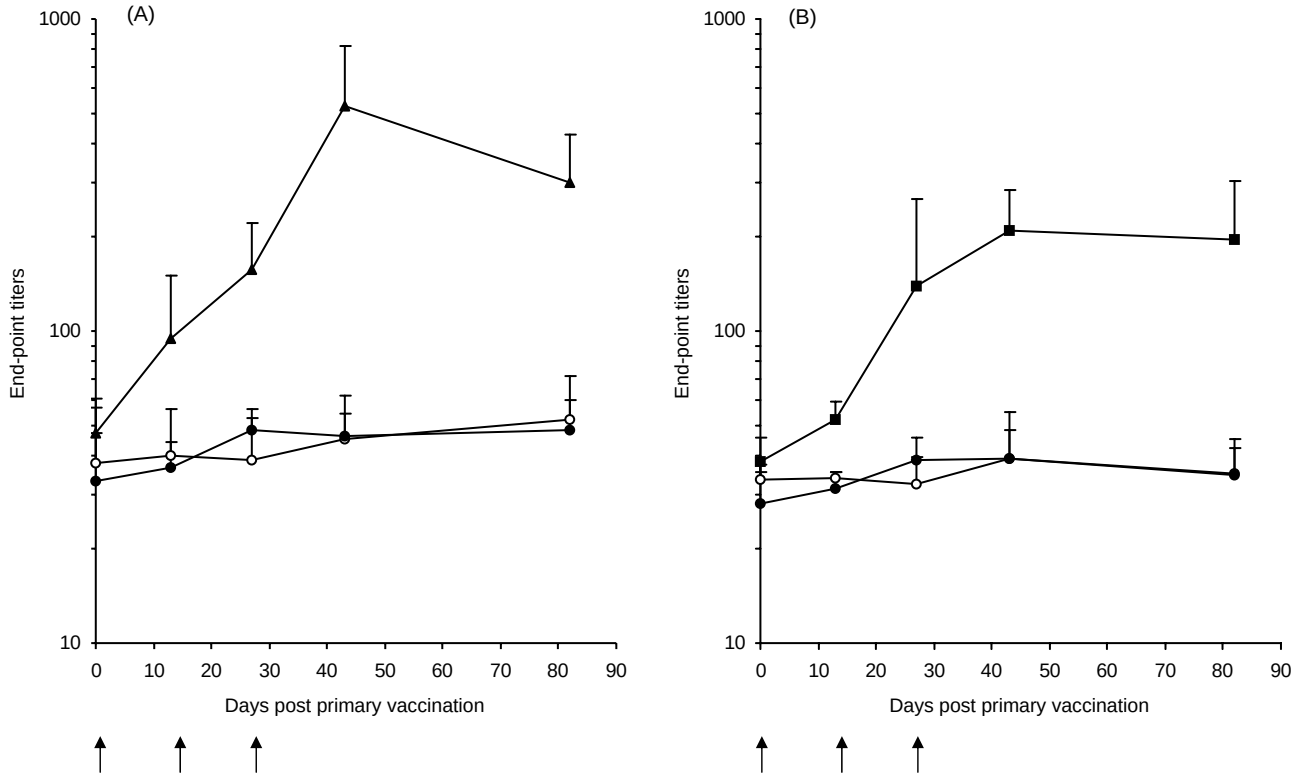

Fig. 5. Serum IgG titers following intra-peritoneal immunisation with recombinant *B. subtilis* spores. Individual samples from groups of eight mice immunised intra-peritoneally (↑) with 1.5×10^9 wild type (●), CotC-TTFC (▲), or CotC-LTB expressing *B. subtilis* spores (■) were tested by ELISA for TTFC- (Panel A) or LTB-specific IgG (Panel B). Sera from a naïve control group (○) were also assayed. The end-point titre was calculated as the dilution of serum producing the same optical density as a 1/40 dilution of a pooled pre-immune serum. Data were presented as arithmetic means ± standard deviations.

anti-LTB responses, these were 10-fold higher than those directed against TTFC. The end-point titres of faecal TTFC- or LTB-specific sIgA were shown to be significantly higher than the control groups ($P < 0.05$) while there was no significant difference between the control groups ($P > 0.05$).

3.7. Analysis of IgG subclasses

We analysed the anti-spore specific IgG subclasses, IgG1, IgG2a and IgG2b, present in the serum following parenteral as well as oral immunisations. The low anti-TTFC and anti-LTB IgG titres did not allow any significant indication of the levels of individual IgG subclasses (data not shown). Following intra-peritoneal immunisations however, analysis of anti-TTFC responses revealed substantial levels of all three subclasses with the IgG2a subclass predominating and appearing first (Fig. 7A). In contrast, anti-LTB responses showed only an IgG2a response with no significant levels of IgG1 or IgG2b (Fig. 7B).

3.8. Prediction of tetanus neutralising antibodies

The presence of neutralising antibodies against tetanus can be measured by a number of in vitro assays as an alternative to in vivo testing [27]. We used the tetanus toxin binding inhibition test (ToBI; [28]) to determine the levels of neutralising IgG TTFC antibodies in groups of mice immunised orally or parenterally with CotC-TTFC spores on the final day post-immunisation (Table 1). As controls we also measured sera from naive mice and mice dosed with non-recombinant (PY79) spores. Finally, we examined

Table 1
Average neutralising antibody units[a]

Route	Group[a]	Dose (μg TTFC per dose)	International units (IU) per ml
Ip[b]	CotC-TTFC (RH114)	0.12	0.017 ± 0.006
	CotB-TTFC (RH103)	0.15	0.014 ± 0.008
	Non-recombinant	–	<0.009
	Naïve	–	<0.009
Oral[c]	CotC-TTFC (RH114)	0.82	0.016 ± 0.006
	CotB-TTFC (RH103)	1.65	0.022 ± 0.011
	Non-recombinant		<0.009
	Naïve		<0.009

[a] Antitoxin levels obtained at the final day of immunisation with recombinant (expressing TTFC) or non-recombinant spores. Expressed in international units/ml.

[b] Mice dosed on days 0, 14 and 28 by the intra-peritoneal route and serum tested at day 82.

[c] Mice dosed on days 0–2, 16–18, 33–35 by intra-gastric gavage and tested at day 68.

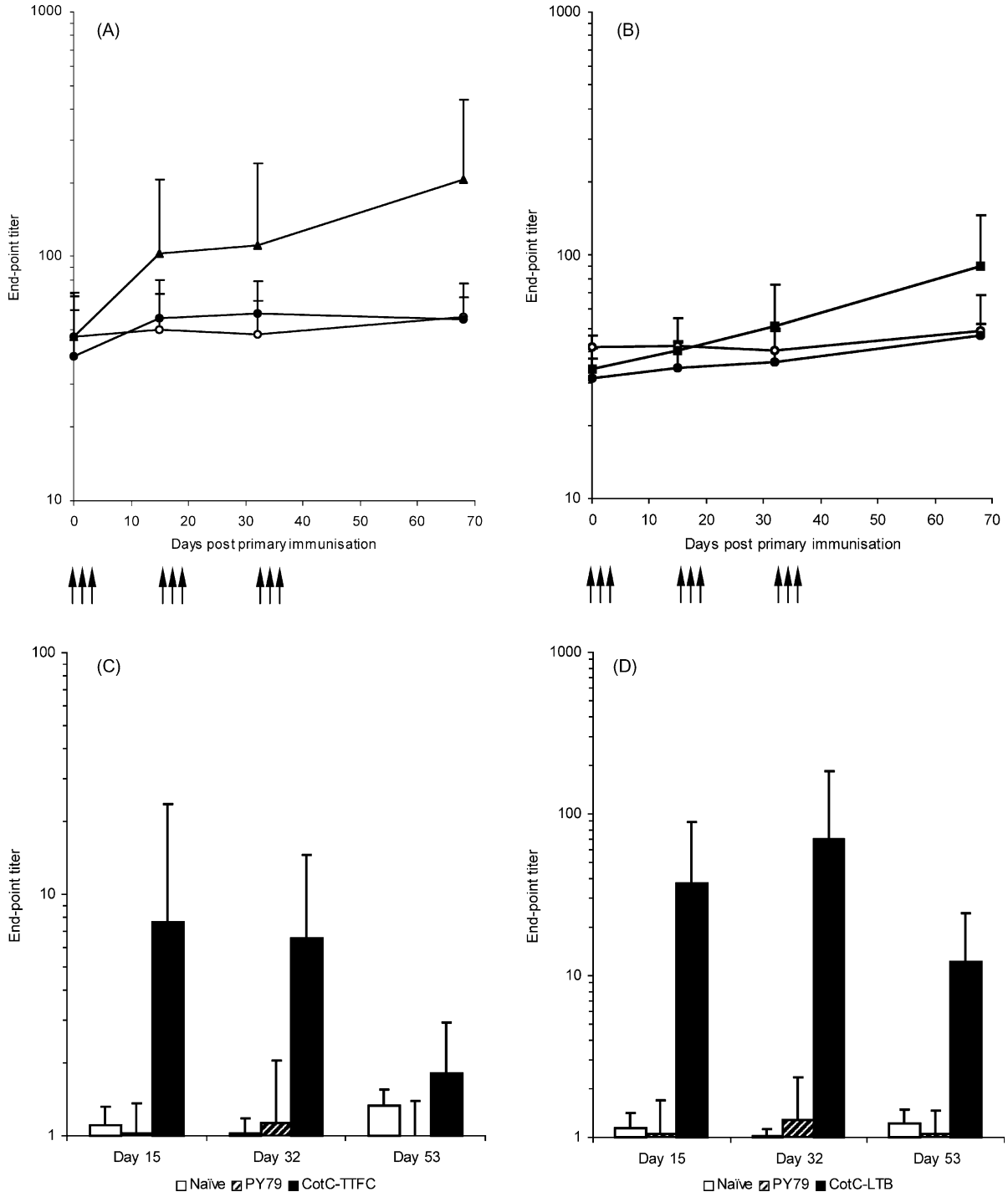

Fig. 6. Serum IgG and faecal sIgA titers following oral immunisation with recombinant *B. subtilis* spores. Groups of eight mice were immunised (↑) orally with spores expressing CotC-TTFC (▲), CotC-LTB (■) or non-recombinant spores (●). A dose of 1×10^{10} spores was used for each oral dose and individual serum samples from groups were tested by ELISA for TTFC- (Panel A) or LTB-specific IgG (Panel B). Sera from a naïve control group (○) were also assayed. Fresh faecal pellets were collected from immunised mice as well as a naïve group and tested for the presence of TTFC- (Panel C) or LTB-specific IgA (Panel D) as described in Section 2. For IgG (Panels A and B), the end-point titre was calculated as the dilution of serum producing the same optical density as a 1/40 dilution of a pooled pre-immune serum. For sIgA (Panels C and D), the end-point titre was calculated as the dilution of the faecal extract producing the same optical density as the undiluted pre-immune faecal extract. Data were presented as arithmetic means ± standard deviations.

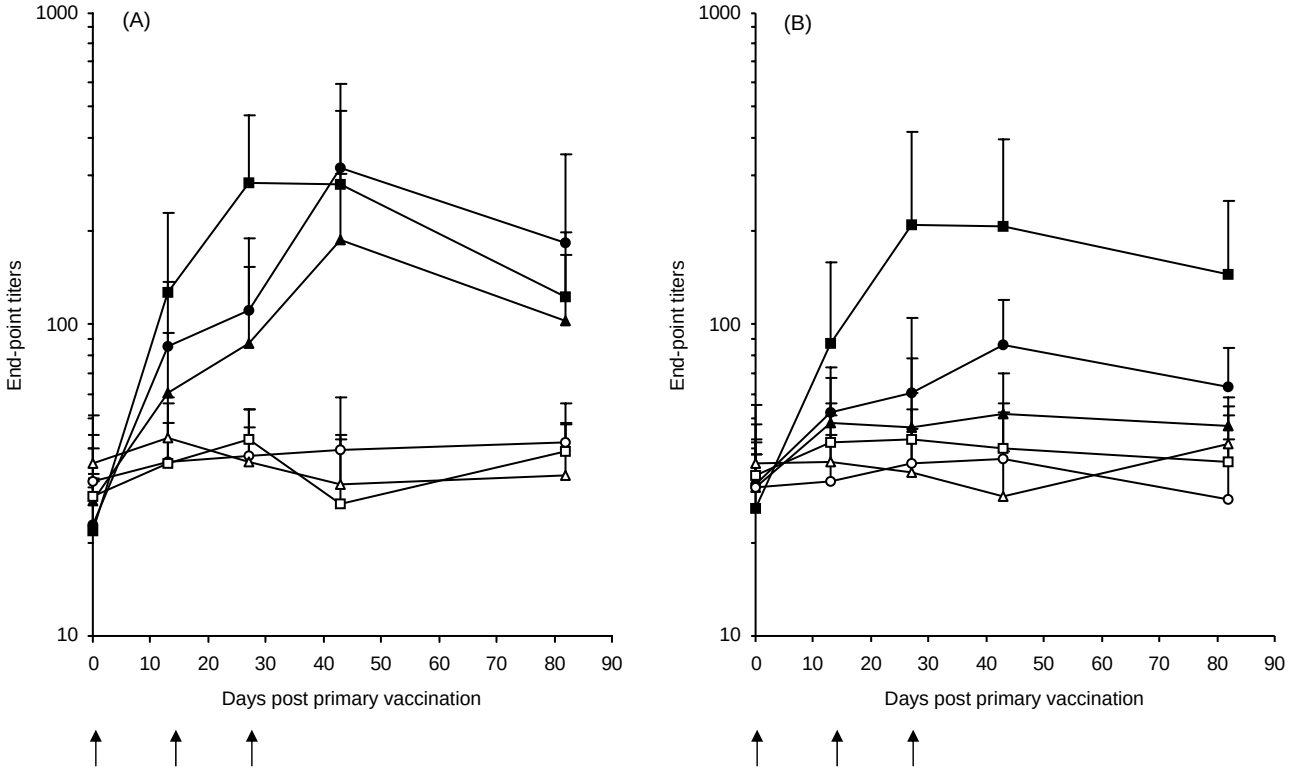

Fig. 7. Serum anti-TTFC and anti-LTB IgG subclasses following parenteral delivery. Sera from naïve and immunised groups (outlined in Fig. 5) were taken at different days post-immunisation (↑) and analysed for IgG1, IgG2a and IgG2b isotypes. Panel A shows anti-TTFC subclasses and Panel B anti-LTB subsclasses. IgG subclasses from mice immunised with spores, IgG1 (●), IgG2a (■) and IgG2b (▲). Naïve groups, IgG1 (○), IgG2a (□) and IgG2b (△). Data were presented as arithmetic means ± standard deviations.

the sera of mice immunised orally and parentally with CotB-TTFC recombinant spores. This was reported recently in a study using C57 BL/6 mice and, most importantly, orally immunised mice were protected against challenge with a 20 LD_{50} dose of tetanus toxin [2]. We established the sensitivity of the test to be 0.009 IU/ml and groups immunised by the oral or intra-peritoneal route gave antitoxin levels above this baseline.

4. Discussion

It has been recently reported that recombinant spores expressing the C-terminal fragment of the tetanus toxin fused to the spore coat protein CotB elicit specific anti-TTFC immune responses following mucosal immunisation of a mouse model as well as protection to a lethal challenge with tetanus toxin [1,2]. This paper expands on this work by showing that it is possible to use at least one other spore coat component, CotC a small 8.8 kDa. polypeptide, to display heterologous antigens, TTFC (51.8 kDa) and LTB (12 kDa). TTFC is non-toxic and immunogenic [21,29–32] and expression in *E. coli* [12], yeast [33], *Salmonella* [34] and *Lactococcus lactis* [21] has been shown to provide protection against tetanus toxin challenge. LTB is the B subunit of the heat-labile toxin produced in enterotoxigenic strains of *E. coli*. LTB has been used extensively for studies of mucosal immunity and oral administration of LTB has been shown to be a potent inducer of serum and mucosal (sIgA) anti-LTB antibodies [5,35–38].

We have found that the presence of either TTFC or LTB on the surface of spores does not significantly affect spore structure and/or function. It would have been possible that when fused to CotC, a large molecule, such as TTFC, may enable surface expression by disruption and protrusion through the coat layers while a smaller antigen, like LTB, may be hidden. Disruption of the apparently, rigid and compact, spore coat layer might damage spore function, affecting either its resistance properties or its ability to germinate correctly. Our data is therefore encouraging and emphasises the flexibility of the spore coat structure and therefore of the spore-based presentation system. The malleability and functional redundancy of both CotC and, in a separate study, CotB [1], makes the spore coat an attractive route for heterologous antigen presentation. One exciting possibility is that, in principle, two different antigens could be displayed simultaneously on the spore surface, using CotC and CotB for presentation.

The second part of this study was to address the immogenicity of the TTFC and LTB monomeric antigens expressed on the spore surface. Our results show that both monomers are immunogenic when delivered parenterally to mice with serum IgG titres that were significantly different from mice receiving non-recombinant spores. This

demonstrates that the TTFC and LTB monomers were stable when displayed on the spore coat. When delivered mucosally CotC-TTFC spores were able to generate both systemic and local anti-TTFC responses. In contrast, systemic IgG responses to LTB were low and barely significant although clear sIgA anti-LTB responses were seen. LTB has been shown to induce strong sIgA responses when given orally and has also been shown to exhibit significant adjuvant activity when administered orally with other antigens [35,39]. Our work implies that when LTB is expressed on the spore it is more efficient at inducing local immunity than systemic, at least with the spore dose and immunisation regime we have chosen here. LTB forms part of the labile toxin of *E. coli* and is made in a pentameric form [40]. In this pentameric form, LTB has adjuvant properties. We reasoned that attached to the CotC protein and displayed on the spore coat it was unlikely that LTB could oligomerise although this was not tested using the in vitro binding assay of LTB to epithelial M1 ganglioside receptors (GM1) [41].

Our subclass analysis also revealed that using the parenteral route different responses to TTFC and LTB were obtained. Th1 cells are involved in cellular immunity while Th2 cells co-ordinate B-cell responses [42,43]. It is generally accepted that a predominance of the IgG2a subclass is indicative of a Th1 response (T-cell responses leading to CTL recruitment as well as IgG synthesis) while the predominance of IgG1 is more indicative of a Th2 response [15,21,29–32]. We found that parenteral immunisation with CotC-LTB spores induced only IgG2a and at relatively high levels. The absence of any IgG1 suggests a biased, Th1 response and involvement of cellular immunity. Confirmation must await analysis of specific cytokine and cellular responses but a straightforward explanation would be that recombinant spores are phagocytosed and that LTB antigens were expressed on the cell surface and indeed IgG2a is the dominant antibody response to a large number of viral infections. Interestingly, when LTB is expressed on the surface of *Streptococcus gordonii* the dominant response following parenteral immunisation was IgG1 inicative of a Th2 response [15]. Responses to TTFC consisted of roughly equivalent amounts of IgG1, IgG2a and IgG2b which could imply a mixed Th1/Th2 response. This mixed response could be unique to spore presentation of the TTFC antigen. Alternatively, release of TTFC, by proteolytic cleavage, from the spore would release soluble antigen which might account for the IgG1 levels. In recent work, we have examined the fate of spores when administered orally to mice [2]. This showed that a small number of intact spores are able to cross the mucosa and are found in the Peyer's Patches. The ability of the whole spore to interact with the GALT is interesting and might be important for cellular immunity.

We did not determine whether the levels of anti-tetanus antibodies obtained in this experiment are protective in vivo but using an established ToBI test, we can state they are certainly within the desired range. In studies using guinea pigs vaccinated by injection, serum tetanus antitoxin levels of between 0.05 and 0.1 IU/ml provided protection against challenge with 50 LD_{50} units of toxin [44]. In a recent study, we have shown that oral immunisation of mice (C57 BL/6) with spores expressing TTFC fused to the spore coat protein CotB (CotB-TTFC) protected seven out of eight mice against a challenge dose of 20 LD_{50} [2]. In the CotB-TTFC study, the actual dose of TTFC was about twice that of TTFC when fused to CotC reported here but the antitoxin levels determined from the ToBI test appear to correlate well with the dose given. We would predict from our data that tetanus antitoxin levels in mice immunised with CotC-TTFC could be protective but to a challenge dose less than 20 LD_{50}.

In conclusion, this study has opened the way for the development of bacterial spores for heterologous antigen presentation and vaccine development. Clearly, the spore has potential for heterologous antigen presentation and ultimately for use as a vaccine system.

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