Available online at www.sciencedirect.com
ScienceDirect
Vaccine 25 (2007) 346–355
Vaccine
www.elsevier.com/locate/vaccine

Immunization against anthrax using *Bacillus subtilis* spores expressing the anthrax protective antigen

Le H. Duc [a,1], Huynh A. Hong [a,1], Helen S. Atkins [b], Helen C. Flick-Smith [b], Zarmina Durrani [c], Sjoerd Rijpkema [c], Richard W. Titball [b,d], Simon M. Cutting [a,*]

[a] *Royal Holloway University of London, Egham, Surrey TW20 0EX, UK*
[b] *Defense Science and Technology Laboratory (Dstl), Porton Down, Salisbury SP4 0JQ, UK*
[c] *National Institute for Biological Standards and Control (NIBSC), South Mimms, Potters Bar EN6 3QG, UK*
[d] *Department Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK*

Received 22 April 2006; received in revised form 7 July 2006; accepted 19 July 2006
Available online 4 August 2006

Abstract

Protective immunity to anthrax can be achieved by antibodies raised against the secreted protective antigen (PA) and this forms the basis of the current acellular vaccines for human use. *Bacillus subtilis* spores have previously been used for delivery of heterologous antigens by the oral and nasal routes and their intrinsic heat-stability make them attractive vaccine vehicles. In this study we have expressed PA, or segments of PA, in *B. subtilis* using two strategies. First, display on the spore coat, and second, in the germinated spore (or vegetative cell). Using parenteral delivery we show that recombinant spores can be used to confer protective immunity in a murine model using an *in vitro* toxin neutralization assay and a challenge experiment with the latter showing protection to 100 median lethal dose of *B. anthracis* spores. PA must be secreted from the live bacterium or alternatively displayed on the spore surface to confer protective immunity. Intracellular expression of PA failed to confer protective immunity. The highest levels of protective immunity were achieved when PA was displayed on the spore surface as well as in the germinating spore.

Keywords: Anthrax; Endospores; Anthrax vaccine; Heat-stable vaccine

1. Introduction

Anthrax caused by *Bacillus anthracis* is now a disease of considerable interest because of its use as an agent of bioterrorism. The disease typically appears in one of three forms: cutaneous, gastrointestinal and pulmonary [1] and two vaccines are currently licensed for human use. In the UK, an alum-precipitated filtrate of a *B. anthracis* Sterne strain culture that is administered by intra-muscular injection is used. In the USA, the AVA (anthrax vaccine adsorbed) is in use consisting mainly of protective antigen (PA) from cultures of the unencapsulated, toxin-producing *B. anthracis* V770-NP1-R strain adsorbed onto aluminum hydroxide and administered by the sub-cutaneous route. Both vaccines have a number of disadvantages including transient reactogenicity associated with the UK vaccine [2–4] and minor reactions at the injection site with the US vaccine [5,6]. Finally, both require frequent boosting. Two virulence factors are produced by *B. anthracis*, the poly-D-glutamic acid capsule [7] and the tripartite toxin that is composed of PA together with edema factor or lethal factor [8]. PA is the essential factor to which protection is conferred in current vaccines [1]. This 83 kDa protein is secreted from the cell and binds to a host cell membrane receptor. Furin-mediated cleavage of a 20 kDa amino-fragment of PA

* Corresponding author. Tel.: +44 1784 443760; fax: +44 1784 434326.
E-mail address: s.cutting@rhul.ac.uk (S.M. Cutting).
[1] These authors contributed equally to this paper.

0264-410X/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.07.037

(referred to as Domain 1a) at the cell surface produces the mature 63 kDa form of PA that carries 4 domains, 1b, 2, 3 and 4. Upon cleavage the 63 kDa cleaved form of PA next forms a heptameric pore to which either the edema factor or lethal factor can bind using domain 1b for interaction [9]. An essential attribute of an anthrax vaccine must therefore be neutralization of PA and prevention of its binding to the host cell surface. Domains 1b and 4 are both known to carry protective epitopes [10] and immunization with recombinant PA has been shown to induce protection against *B. anthracis* infection [10,11].

Bacterial endospores have shown potential as vehicles for delivery of heterologous antigens with proof-of-principle studies demonstrating that orally delivered *Bacillus subtilis* spores expressing a *Clostridium tetani* antigen on the spore surface can protect mice against toxin challenge [12]. Other studies have shown that the *B. subtilis* spore can germinate in the murine gut and that this provides an additional route for antigen delivery [13,14]. In this approach the antigen is expressed in the vegetative cell and relies upon germination of the spore in the gastrointestinal tract (GIT). Interestingly, it has also been shown that spores given orally can disseminate to the gut-associated lymphoid tissue (GALT) and enter the Peyer's Patches and mesenteric lymphoid tissues [12,15]. Although this is likely to represent the fate of only a subpopulation of ingested spores phagocytosed spores have been shown to germinate and persist within the phagosome before being destroyed [16]. Like *B. anthracis*, *B. subtilis* can cross the gut wall and spores can germinate in the phagosome. However, unlike *B. anthracis*, *B. subtilis* is unable to establish a productive infection or to cause disease. The heat-stability of spores coupled with a convenient route of delivery (e.g., oral) make spore vaccines promising candidates for use in developing countries and for military personnel.

We have considered that *B. subtilis* could provide a potential method for immunizing against anthrax. *B. subtilis* is a non-pathogen and is being used commercially as a probiotic [17]. Although the importance of cellular responses in anthrax immunity are currently poorly understood [6,18] this feature could be important. Accordingly, we have engineered *B. subtilis* to express the *B. anthracis* protective antigen on the spore surface and/or from the germinating spore as a secreted protein. In this study we evaluate the systemic responses produced to these spores given to mice by a parenteral route and show that mice can be protected against a lethal dose of, *B. anthracis,* STI spores (100 median lethal dose [MLD]).

2. Materials and methods

2.1. Bacterial strains and transformation

The laboratory strain of *B. subtilis* used in this work was strain PY79 a prototrophic, Spo$^+$, derivative of the type strain 168 [19]. All recombinant strains described here are isogenic derivatives of PY79. Plasmid amplification for nucleotide sequencing, subcloning experiments and transformation of *Escherichia coli* competent cells were performed in the *E. coli* strain TG1 [20]. Methods for growth, sporulation and transformation of *B. subtilis* were detailed elsewhere previously [21]. *B. anthracis* STI (Tox$^+$ Cap$^-$) was used for the challenge experiments.

Table 1
B. subtilis recombinant strains[a]

Strain no.	Relevant genotype	Expressed PA antigen and location[b]		Quantification[c]
		Vegetative cell	Spore	
DL330	*thrC::PrrnO-pagA1b-3*	Domains 1b-3 50 kDa		3.0×10^{-4} pg/cell
DL346	*thrC::PrrnO-pagA*	Full length PA[d] secreted 83 kDa		53.6 μg/ml culture supernatant
DL348	*thrC::PrrnO-pagA4*	Domain 4, 19 kDa		4.5×10^{-4} pg/cell
DL359	*thrC::PrrnO-pagA1-4*	Full length PA[d] 85 kDa		1.4×10^{-3} pg/cell
DL372	*amyE::cotC-pagA4*		CotC-domain 4 31 kDa	2.2×10^{-4} pg/sp
DL374	*amyE::cotB-pagA1b-3*		CotB-domains 1b-3 91 kDa	1.5×10^{-4} pg/sp
DL387	*amyE::cotBΔ-pagA4*		CotB-domain 4 48 kDa	2.4×10^{-4} pg/sp
DL507	*thrC::PrrnO-pagA, amyE::cotB-pagA1b-3*	Full length PA[d] secreted 83 kDa	CotB-domains 1b-3 91 kDa	53.6 μg/ml culture medium 1.5×10^{-4} pg/sp
DL509	*thrC::PrrnO-pagA, amyE::cotC-pagA4*	Full length PA[d] secreted 83 kDa	CotC-domain 4 31 kDa	53.6 μg/ml culture medium 2.2×10^{-4} pg/sp
DL511	*thrC::PrrnO-pagA, amyE::cotBΔ-pagA4*	Full length PA[d] secreted 83 kDa	CotB-domain 4 48 kDa	53.6 μg/ml culture medium 2.4×10^{-4} pg/sp

[a] Strains were made as described in Section 2.
[b] Molecular weight of expressed protein chimera determined by SDS-PAGE analysis.
[c] Verified by Western blotting and quantified by dot-blot analysis on exponentially growing vegetative cells, filtered culture supernatants or coat extracts from mature spores (sp).
[d] There are two versions of full length PA. The secreted version of 83 kDa where the N-terminal leader sequence is cleaved following membrane transition and an 85 kDa that lacks the leader sequence and cannot be secreted. The higher molecular weight. reflects a 2.3 kDa leader introduced during cloning (see Section 2).

2.2. Recombinant strains expressing heterologous antigens

2.2.1. PrrnO expression vectors

Strains made are listed in Table 1. The first stage was to construct a vector, pDL242, for expression of the cloned DNA under the control of the PrrnO promoter (Fig. 1A). The *rrnO* promoter was first amplified by PCR using *B. subtilis* strain PY79 chromosome as template DNA and two oligonucleotides (gaagatctGCATGACCATTATGACTAG and gctctaga-ACAGGTTAAGTTCACCGCATCC) as primers, resulting in a 244 bp amplicon containing the −35 and −10 regions of the *B. subtilis rrnO* promoter. The ribosome binding site (RBS) of the *B. subtilis sspA* gene was artificially synthesized by annealing two complementary oligonucleotides (ctagaACAAGGAGGTGAGACc and catggGTCTCACCTC-CTTGTt). The *rrnO* promoter amplicon was restricted using *Bgl*II and *Xba*I enzymes, ligated to the *sspA* RBS and then cloned into plasmid pET28b (Novagen) between the *Bgl*II and *Nco*I restriction enzyme sites, yielding plasmid pDL180. The region containing the *rrnO* promoter-*sspA* RBS-pET28b multiple cloning site (MCS) was amplified from pDL180 using gccagctgCGATGCGTCCGGCGTAGAGGATCG and gccagctgGCAGCCGGATCTCAGTGG TGGTGG oligonucleotides as primers, and the PCR product, blunt-ended using the *Pvu*II restriction enzyme was cloned into plasmid pDG1664 [22] between *Eco*RI and *Bam*HI restriction enzyme sites which had been blunt-ended using Klenow fragment, yielding plasmid pDL242. To express PA or fragments of PA the *pagA* ORFs corresponding to different domains of PA were PCR amplified from *B. anthracis* pXO1 and ligated, in frame, into the MCS of pDL242 (Fig. 1A). The clone was then verified by DNA sequencing. The leader sequence (black box in Fig. 1A) added approximately 23 amino acids to ORFs cloned into the MCS after the *Nhe*I site and enabled secretion of the nascent polypeptide across the cytoplasmic membrane. To introduce the cloned DNA into the *B. subtilis* genome the pDL242 derivative was subsequently linearized by digestion with *Pvu*I and transformed into competent cells of strain PY79 with selection for erythromycin resistance (Erm^R; 1 μg/ml). Erm^R transformants were checked for their ability to grow on minimal agar plates or on plates supple-

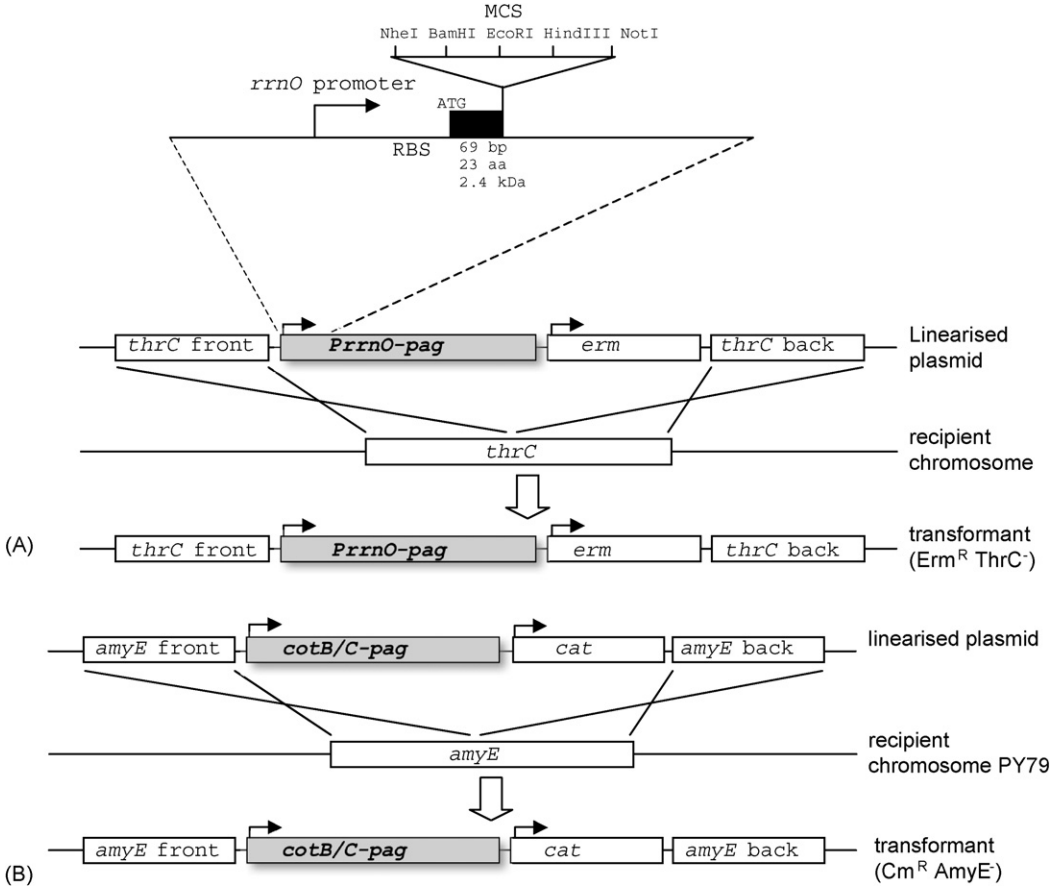

Fig. 1. Cloning strategy. Panel A shows the essential components of the pDL242 cloning cassette. DNA is cloned into the multiple cloning site (MCS) that enables in frame fusion to a short 23 residue leader sequence under the control of the *B. subtilis rrnO* promoter and the ribosome binding site (RBS) of the *B. subtilis sspA* gene. The plasmid cassette is then linearized and introduced into competent cells of the recipient. Selection for Erm^R resistance (erythromycin resistance) enables a double-crossover recombination between the front and back portions of the *thrC* gene as shown and described elsewhere [22]. Panel B shows the integration of linearised pDG364 DNA carrying a chimeric gene at the *amyE* locus following selection for Cm^R encoded by the *cat* gene (chloramphenicol acetyltransferase).

mented with threonine as described [22]. Erm^R transformants that were *thrC*$^-$ carried stable integrations of the *PrrnO-pagA* clones as shown in Fig. 1A.

2.2.2. Expression vectors expressing PA domains fused to a spore coat protein

Three *B. subtilis* strains, termed DL374, DL387 and DL372, were made that displayed domains of PA on the spore surface (Table 1). DL374 carried domains 1b-3 of PA fused to the full length CotB protein (comprising 382 amino acids), DL387 carried domain 4 fused to a truncated CotB protein (fused after residue 277 of CotB), and DL372 carried domain 4 fused to the full length CotC protein (comprising 120 amino acids). In all cases, fusions were C-terminal. Initially, PCR was used to amplify the *pagA* fragments from pXO1 that were cloned in frame to *cotB* or *cotC* sequences contained in pBluescript. The *cotB* and *cotC* sequences carried the natural *B. subtilis* promoter ensuring expression of the chimera only during development. Clones were verified by DNA sequencing and then subcloned into the plasmid pDG364, allowing integration of cloned DNA at the *amyE* (amylase) locus of *B. subtilis* as shown in Fig. 1 [22]. Competent cells of PY79 were transformed with pDG364 clones that had been linearized by digestion with *Pvu*II. Selection was made for chloramphenicol resistance (Cm^R; 5 μg/ml) and then checked for their ability to produce amylase. Transformants that were Cm^R and Amy$^-$ were then checked for expression of PA on the spore surface.

2.2.3. Strains containing two expression cassettes

Three strains of *B. subtilis* were made that carried two expression cassettes using a simplified procedure of transformations (Table 1). Thus, PA or fragments of PA could be expressed both within the vegetative cell and on the spore coat within each strain. For example, strain DL507 was made by transforming competent cells of strain DL346 (*thrC::rrnO-pagA*) with plasmid pDG364 carrying the recombinant *cotB-pagA1b-3* followed by selection for Cm^R and screening for transformants that were Amy$^-$.

2.3. Recombinant PA protein

Recombinant PA was produced in *E. coli* BL21 (DE3) from a pET28b expression vector (Novagen) that carried the *pagA* gene fused to a C-terminal poly-histidine tag. High levels of expression were obtained upon induction with IPTG, and PA was purified by nickel affinity column chromotography. Eluted PA-His protein was checked for integrity by SDS-PAGE and the concentration determined using the Bio-Rad DC Protein Assay kit.

2.4. Mouse anti-PA antibody

Purified recombinant PA was used to raise anti-PA antibody in Balb/C mice. On day 0, 6-week-old mice were injected via the intra-peritoneal route with 10 μg PA in sterile 0.85% NaCl formulated with 50% (v/v) Freund incomplete adjuvant in a total volume of 100 μl per dose. The animals were boosted on days 14 and 28, and sera were collected on day 42. Mouse sera were checked by Western blotting and ELISA, and used in immuno-blotting experiments at a dilution of 1:1000 followed by a anti-mouse IgG-HRP conjugate (Sigma) at a dilution of 1:3000.

2.5. Preparation of spores

Sporulation of each *B. subtilis* strain was made in DSM (Difco-Sporulation Medium) using the exhaustion method as described elsewhere [23]. Using this method we could reliably produce 6×10^{10} spores per litre of DSM. Each batch of spores prepared in this way was checked for the presence of the CotB/CotC-PA fusion proteins in extracts of spore coat protein or the expression of PA protein in growing vegetative cells by Western blotting using a polyclonal anti-PA antiserum.

2.6. Extraction of proteins from recombinant spores and vegetative cells

Spore coat proteins were extracted from suspensions of spores at high density (>1×10^{10} spores/ml) using an SDS-DTT extraction buffer [23]. Extracted proteins were assessed for integrity by SDS-PAGE and for concentration by the BioRad DC Protein Assay kit (BioRad). For vegetative cell expression, cells were grown to early stationary phase (approximately 4 h at 37 °C), centrifuged and the culture supernatant was kept for analysis.

2.7. Western and dot-blot analysis

Western blot filters were visualized by the ECL (Amersham Pharmacia Biotech) method following the manufacturer's instruction. Serial dilutions of spore coat extract, vegetative cell culture supernatant and purified PA were used for dot blot analysis. Filters were visualized by the ECL (Amersham Pharmacia Biotech) method and subjected to densitometric analysis by the ChemiDoc XRS system equipped with the Quantity One software (BioRad). Standard curves were generated from known amounts of purified PA, from which the concentration in the unknown samples were estimated.

2.8. Confocal immunofluorescent microscopy

The expression and assembly of CotB-PA1b-3 on the spore coat was confirmed using confocal immunofluorescent microscopy and a polyclonal antiserum to PA. Cells of recombinant DL374 or wild type PY79 strains were allowed to sporulate by the resuspension method [23] and samples were taken and prepared for microscopy as described previously [12].

2.9. Immunisations

Groups of six mice (female, Balb/C, 6 weeks old) were immunised by the intra-peritoneal route with pure spore suspensions of either recombinant strains or the control strain PY79. Injections contained 1×10^9 spores/ml in a volume of 0.1 ml administered on days 0, 14 and 28. A naïve, non-immunised control group or a group immunised with purified rPA protein (10 μg/injection, with 1 mg/ml Alhydrogel [Sigma] as adjuvant) were also included. Three doses of rPA (10 μg) have been shown to be protective in A/J mice [10]. Serum samples were taken on days −1, 13, 27 and 44. A second set of immunisations was carried out for the intra-peritoneal challenge with *B. anthracis* spores (see below).

2.10. Indirect ELISA for detection of PA-specific serum antibodies

Serum samples from mice were tested by ELISA using the procedure described by Duc et al. [12] with the following modifications: plates were coated with 1 μg/ml purified PA, blocked with 2% BSA for 1.5 h, and serum samples were incubated for 1 h at 37 °C. Dilution curves were drawn for each sample and endpoint titers were calculated as the reciprocal of the dilution producing an optical density that was 0.1 U greater than the background value (1/40 dilution of a pooled pre-immune serum). Titres lower than 40 (negative samples) were arbitrarily assigned as 40. Statistical comparisons between groups were made by the Mann–Whitney *U* test. For multiple comparisons among groups, the Mann–Whitney *U* test was carried out for each paired group. A *P* value of >0.05 was considered non-significant.

2.11. Toxin neutralising assay (TNA)

Sera from mice were subjected to the toxin neutralising assay using the protocol described and validated by Herring et al. [24] with the following modifications. The murine macrophage-like cell line RAW264.7 (European Collection of Cell Cultures, ECACC) was used instead of J774A.1 (the RAW264.7 macrophage cell line is also sensitive to the anthrax lethal toxin and has been used by others in TNA, e.g. [25]). The anthrax lethal toxin (LeTx) consisted of 0.074 μg of LF and 0.093 μg of PA (List Biological Laboratories) per ml in cell culture medium. To measure the viability of macrophage cells, WST-1 cell proliferation reagent (Roche Diagnostics) was used according to the manufacturer's manual. TNA assays were performed independently at the NIBSC and RHUL using the same protocol. At each site the assay was done in triplicate for each serum sample. A dilution curve was drawn from each of the triplicates and endpoint titer was calculated as the reciprocal of the highest dilution of serum sample which gave absorbance higher than the median absorbance of control wells (medium only + LeTx only)/2. Initial dilution of each sample was 1/40, and titres lower than 40 (negative samples) were arbitrarily assigned as 1. Results were presented as arithmetic mean titres as measured at both institutions. Statistical comparisons between groups were made using the Mann–Whitney *U* test. For multiple comparisons among groups, the Mann–Whitney *U* test was carried out for each group pair. A *P* value of >0.05 was considered non-significant.

2.12. Intra-peritoneal challenge with B. anthracis spores

The correlation of serum IgG titers and TNA responses between mice receiving rPA and DL507 spores suggested that DL507 could induce protection. Groups of eight female A/J mice (8–12 weeks old) were immunized with 1×10^9 spores of DL507 or PY79 strains on days 0, 14 and 28. Control groups included naïve, non-immunized, mice, or groups of mice immunized with rPA (10 μg/injection). Serum samples were taken on day 63 and, on day 72 mice were challenged with approximately 1×10^5 STI (Tox^+ Cap^-) spores (equivalent to 100 50% median lethal doses (MLD_{50}s)) [18] by the intra-peritoneal route. The animals were observed everyday for 14 days to determine their protected status. The challenge experiment was carried out in a blinded fashion and humane endpoints were strictly observed so that any animal, which displayed a collection of clinical signs that indicated a lethal infection was culled and death was recorded. Individuals showing no symptoms after 14 days were considered immune.

3. Results

3.1. Expression of PA in vegetative cells and on spores of B. subtilis

The *pagA* gene, or gene fragments encoding domains of PA, were cloned in *B. subtilis* using vectors pDL242 or pDG364 (Section 2 and Fig. 1) to facilitate expression of heterologous genes within the vegetative cell or on the spore surface. For vegetative cell expression, DNA was cloned, in frame, with a short leader sequence of 23 codons (2.4 kDa) that was under the control of the *B. subtilis* PrrnO promoter and the *sspA* RBS to provide maximal levels of expression of cloned DNA during vegetative cell growth. Using this strategy a number of different *pagA* clones were made in *B. subtilis* (Table 1). These expressed full length PA (DL346), domains 1–4 (DL359), domains 1b-3 (DL330) and domain 4 (DL348). In the case of DL359 the first 29 residues of *pagA* gene had been deleted, in frame, so that PA was synthesised without the N-terminal 29 amino acid (3.2 kDa) secretory signal sequence that is cleaved following translocation of PA across the membranes.

The levels of PA produced in vegetative cell growth were verified by Western blotting and quantified by dot blotting (Table 1). Western blotting results are shown for DL346 and DL359 (Fig. 2A) and demonstrate that the 83 kDa PA protein

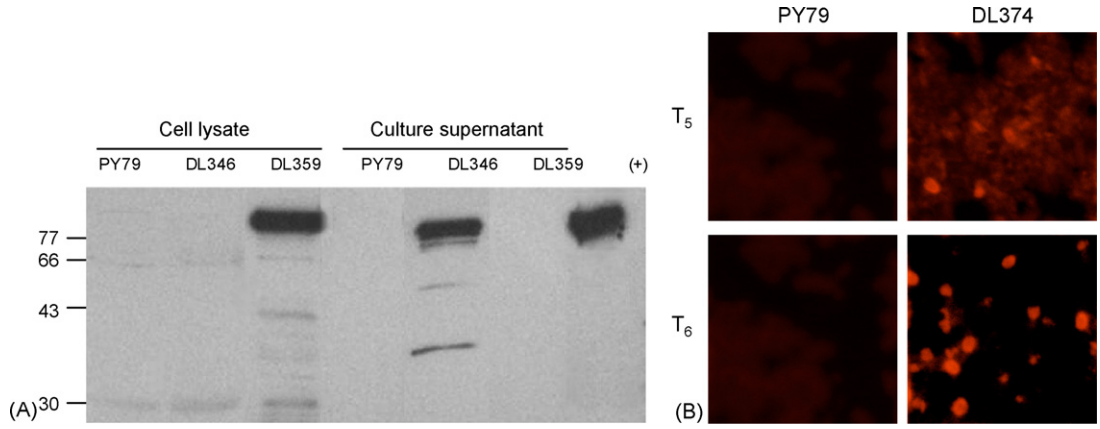

Fig. 2. Immuno-detection of PA expression. Panel A: *B. subtilis* strains DL346, DL359 and PY79 (non-recombinant control) were grown in LB medium and, at exponential phase (OD_{600nm} = 0.8), total cell lysates and cell culture supernatant examined by SDS-PAGE followed by Western blotting. For total cell lysates, 10 ml of culture was centrifuged and suspended in 250 μl of SDS-PAGE loading buffer and 20 μl loaded on a SDS-PAGE gel (12.5%). For culture supernatants, the supernatant was filtered (0.45 mm) and 15 μl mixed with 5 μl of a 4× loading dye and examined by SDS-PAGE. After blotting, membranes were probed with mouse polyclonal antiserum to PA (1:1000 dilution) followed by a 1:3000 dilution of an anti-mouse-IgG-HRP conjugate. (+) is purified PA (0.7 μg). Panel B. *B. subtilis* strain DL374 and PY79 were induced to sporulate by the resuspension method and samples taken 5 h (T_5) and 6 h (T_6) after the onset of development. Samples were labelled with mouse anti-PA serum followed by the anti-mouse IgG-TRITC conjugate. Images were taken by a Nikon Eclipse fluorescence microscope equipped with a BioRad Radiance 2100 laser scanning system. The image size was 16μm × 16 μm.

was secreted in DL346 cells but not in DL359 cells. The higher molecular weight of the PA protein in DL359 cells is due to the 2.4 kDa leader sequence added in the cloning procedure. Interestingly, these results show that even with a 23 amino acid leader sequence (including six histidine residues) attached to the N-terminus of PA the protein does not prevent secretion of PA across the membrane.

In addition to expression in the vegetative cell, the spore coat was also used for antigen display with either CotB (43 kDa) or CotC (15 kDa) as anchors. The use of these proteins for antigen display has been described previously [26,27]. Domain 1b-3 was fused to the C-terminus of CotB (DL374) and domain 4 was fused to a truncated CotB protein (DL387) or full length CotC (DL372). Expression of these PA domains was verified by Western blotting (not shown) and confocal imaging of immunostained spores (Fig. 2B shows surface display of D1b-3 in DL374). Finally, we made three *B. subtilis* strains containing two expression cassettes as listed in Table 1. These strains expressed one of the three chimeric proteins on the spore coat (CotB-PA1b-3, CotBΔ-PA4, or CotC-PA4) as well as the full length, secretable, form of PA in the vegetative cell.

3.2. Serum antibody responses following immunisation with recombinant spores

The immunogenicity of the recombinant spores was monitored after intra-peritoneal delivery into groups of six Balb/C mice. All of the recombinant *B. subtilis* strains expressing full-length PA or domains of PA whether in the vegetative cell or displayed on the spore surface were able to trigger significantly higher serum anti-PA IgG responses ($P < 0.05$) than those of naïve non-immunised mice or mice immunised with non-recombinant PY79 spores as assessed by ELISA (Fig. 3). Antibody titres in all groups receiving recombinant spores were at levels close to those obtained in mice receiving three injections of rPA (10 μg/injection). The temporal profile of anti-PA IgG responses generated against DL346, DL374 and DL507 spores is shown in Fig. 4 and revealed that DL507 and DL346 spores generated responses similar to injection with purified rPA protein ($P > 0.05$). Responses derived from injection of DL374 spores generated lower IgG responses compared to those of DL507 and DL346 spores ($P < 0.05$), however this IgG level remained higher than those of naïve non-immunised group or group receiving non-recombinant PY79 spores ($P < 0.05$). In all cases though, after the third injection on day 28 the steady increase in total IgG responses began to level-off.

3.3. Analysis of IgG subclasses

Sera from day 44 were examined for the presence of PA-specific IgG1, IgG2a and IgG2b subclasses (Fig. 3). In mice immunised with purified PA protein, the mean titers were significantly higher than baseline titers in naïve mice and control mice immunised with non-recombinant PY79 spores ($P < 0.05$), with a predominance of the IgG1 subclass followed by IgG2b and then IgG2a. By contrast animals immunised with DL372, DL374 and DL387 recombinant spores showed high levels of the IgG1, IgG2a and IgG2b isotypes with IgG1 being the most abundant subclass and IgG2b the least, significantly different from naïve mice and group immunised with PY79 spores ($P < 0.05$). This was also true for mice immunised with DL346, DL507, DL509 and DL511. However, in mice immunised with DL328, DL330, DL348 or DL359 spores where PA was only expressed upon spore germination and was not secreted only the IgG2a subclass was produced in significant amounts ($P < 0.05$). No

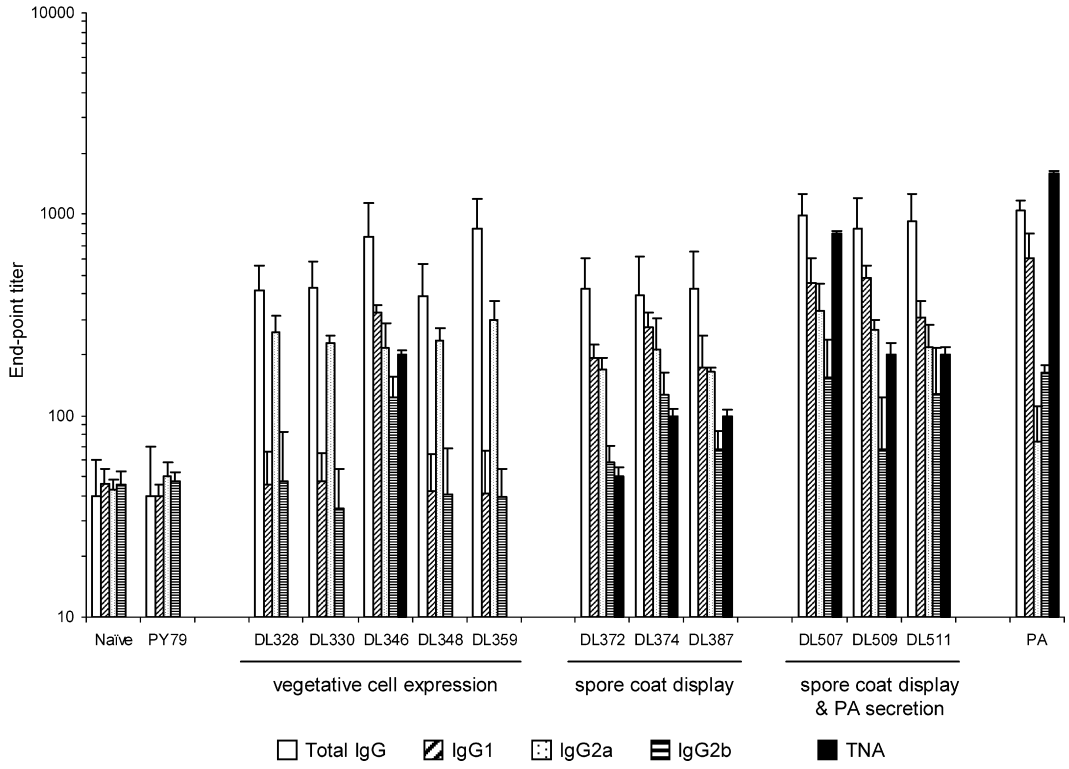

Fig. 3. Immunogenicity of PA expressed in recombinant *B. subtilis* strains. Groups of six Balb/C mice were immunised by the intra-peritoneal route with 1×10^9 spores of recombinant *B. subtilis* strains (Section 2 and Table 1) on days 0, 14 and 28. Serum samples were collected on day 44 and tested by ELISA for PA-specific total IgG (on individual samples with data presented as arithmetic means), by the TNA for ability to neutralise anthrax lethal toxin (on pooled samples of each group). Anti-PA antibody isotype profiles (IgG1, IG2a, IgG2b) isotypes were also determined by indirect ELISA. Sera from a naïve control group were also assayed. Data are presented as arithmetic means and error bars are standard deviations.

significant variation ($P > 0.05$) in any of the subclasses was seen between groups administered with non-recombinant spores and the naïve group.

3.4. Export or surface display of PA is required for the induction of toxin neutralising antibody

The anthrax lethal toxin neutralising assay (TNA) was used to determine the levels of neutralising IgG antibodies in groups of mice immunised parenterally with recombinant spores on day 44 of the immunisation schedule (Fig. 3). As controls, we also measured sera from naive mice, mice immunised with non-recombinant PY79 spores, or with purified rPA protein (three doses; 10 μg/injection). No neutralising antibodies were detected in the naïve group or in the group immunised with PY79 spores. Mice dosed with rPA had serum antibody titres greater than 10^3. For mice immunised with spores where PA was expressed only in the vegetative cell (i.e., following spore germination), toxin neutralising antibodies were only generated when PA was secreted (strain DL346) and in strains DL328, DL330, DL348 and DL359, no measurable levels of neutralising antibody could be detected. Neutralising antibody levels, when detected, were significantly higher than those of the naïve group or the group immunised with PY79 spores ($P < 0.05$). When PA was expressed on the spore coat as well as secreted (strain DL507), the neutralising antibody level was significantly increased compared to when PA was either expressed on the spore coat only (in strains DL372, DL374 and DL387) or by secretion only (in strain DL346) ($P < 0.05$).

In mice immunised with spores that displayed domains 4 (DL372 or DL387) or 1b-3 (DL374) on the spore surface toxin neutralising antibodies were found to be detectable at significant levels compared to the naïve group or group immunised with PY79 spores ($P < 0.05$). However these levels remained significantly ($P < 0.05$) lower than when PA was secreted from the vegetative cell (DL346). As shown in Fig. 3, in mice immunised with spores (DL507, DL509 and DL511) which expressed PA domains on the spore surface as well as PA secreted from the vegetative cell, TNA titres were significantly enhanced in all cases compared to without secreted PA, particularly in the case of DL507 ($P < 0.05$).

3.5. Challenge with B. anthracis spores

The high levels of neutralising antibody titers determined from the TNA in mice immunised with DL507 spores suggested these might be at protective levels [10]. ELISA and TNA titres of pre-challenge sera (day 63) of A/J mice were comparable to those of final sera (day 45) of Balb/C mice (data not shown). In order to test the biological activity of the elicited antitoxin response and the associated level of pro-

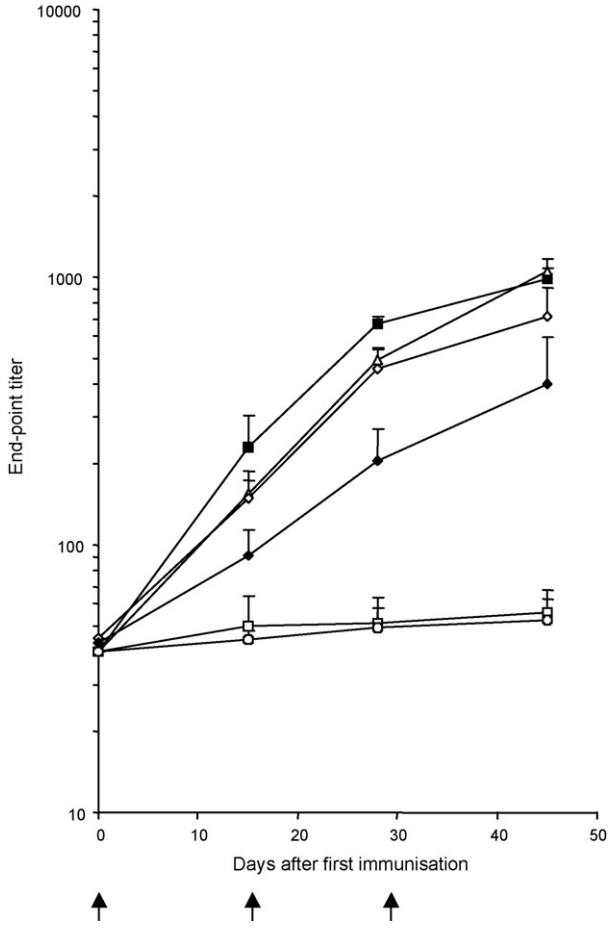

Fig. 4. Serum anti-PA IgG responses following parenteral delivery of *B. subtilis* spores. Groups of six Balb/C mice were immunised (↑) by the intra-peritoneal route with 1×10^9 spores of DL507 (■), DL346 (◊), DL374 (♦) or non-recombinant spores (PY79; □). Individual serum samples from groups were tested by ELISA for PA-specific IgG. Sera from a naïve control group (◯) and a group immunised with 10 μg/dose of purified PA protein (△) were also assayed. Data are presented as arithmetic means and error bars are standard deviations.

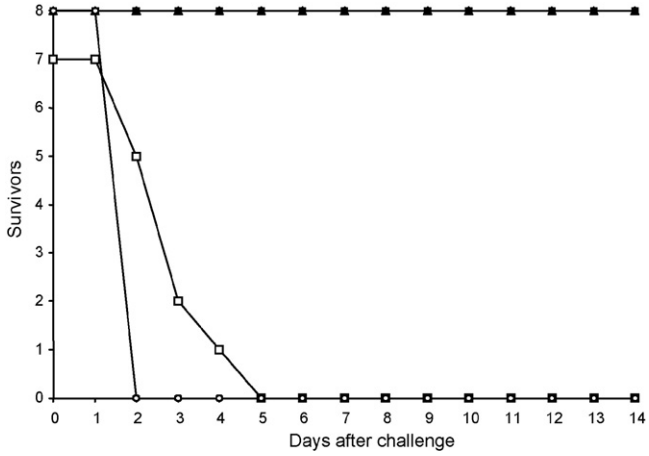

Fig. 5. Protection after intra-peritoneal challenge with *B. anthracis* spores Groups of eight A/J mice were immunised by the intra-peritoneal route with 1×10^9 spores carrying *cotB-pagA1b-3 rrnO-pagA* (DL507; ■) or non-recombinant spores (PY79; □) on day 0, 14 and 28. On day 72 mice were challenged with 100 MLD of *B. anthracis* STI spores via the intra-peritoneal route, and numbers of survivors were recorded. A naïve control group (◯) and a group immunised with recombinant PA protein (△; 10 μg/dose) were included.

tection, A/J mice immunised with DL507 spores were challenged with a dose of approximately 100 MLD of *B. anthracis* STI spores given by the intra-peritoneal route (Fig. 5). Mice are generally considered a poor model for anthrax infection but A/J mice were used here because they are uniquely susceptible to the *B. anthracis* STI strain, which is capsule deficient [28]. In addition, A/J mice have previously been used to assess the efficacy of PA based vaccines against anthrax [29,30]. These mice were fully protected against this challenge. In comparison, all naïve mice and mice immunised with wild type *B. subtilis* spores (PY79) showed anthrax symptoms and died within 2–5 days after the challenge.

4. Discussion

In this study, we have questioned whether the *B. subtilis* spore could be used to deliver PA either by display on the spore surface or by germination of the spore within the host animal and its subsequent expression within the germinating spore, i.e., the live vegetative cell. In previous work PA has been expressed in *B. subtilis* primarily to evaluate its use as a host expression system to produce rPA and thereby improve the safety of the existing anthrax vaccines [31,32]. In these studies, PA has been detected in culture supernatants and shown to confer protection in guinea pigs and in mice immunised with vegetative cells [31,32]. These studies also indicated that the *B. subtilis* spore could be used for PA delivery and for this reason we have examined in detail the potential use of the *B. subtilis* spore as a vaccine delivery system against anthrax. *B. subtilis* is particularly amenable to genetic manipulation and has been used for heterologous antigen display and oral vaccination against tetanus and offers a promising route for vaccination against toxin-based diseases [12,14]. The outcome of our studies reported here, are that the *B. subtilis* spore can be used for efficient delivery of the PA polypeptide by a parenteral route and can be used to confer protection in a murine model. Importantly, though, these studies also reveal a number of important aspects of using recombinant *B. subtilis* spores for anthrax immunisation.

Firstly, we have shown that to induce protective, i.e., neutralising, antibodies PA must be expressed outside of the delivery vehicle, either by secretion from the vegetative cell or by display on the spore surface. Optimal expression appears to require expression and secretion of the full-length PA polypeptide and interestingly, up to 23 amino acids can be added to the N-terminus with no apparent effect on its ability to target and translocate the cytoplasmic membrane of *B. subtilis*. Although high levels of IgG antibodies are produced against PA in cells that don't secrete PA (e.g., DL330, DL348 and DL359), in mice immunised with these strains

no neutralising antibodies were detectable. A straightforward explanation to account for these non-neutralising anti-PA IgG titres is that they are antibodies raised against incorrectly folded, or unfolded, PA polypeptide. Normally, PA would be synthesised in the cytoplasm and then be secreted. Following secretion, the N-terminal signal sequence would be cleaved and the nascent polypeptide refolded into its correct form. Antibodies made against secreted PA would therefore be protective but it is quite possible that antibodies raised against intracellular, unfolded, PA would fail to recognise the appropriate neutralising epitopes of the folded protein. Of course, if true, this must also imply that domains 1b-3 and domain 4 of PA displayed on the spore surface (i.e., in spores of DL372, DL374 and DL387; see below) must be presented properly. Interestingly, only the IgG2a isotype is generated against cells expressing PA intracellularly with no detectable levels of the IgG1 or IgG2b subclasses that is potentially indicative of a type 1 (Th1) T-cell response [33–35]. It is not clear whether the export of PA is required for the induction of protective antibodies in other vaccine vector systems. However, the protection against anthrax afforded after immunisation by recombinant *Salmonella enterica* was also improved by exporting PA using the *hly* export system [36].

The TNA test is, of course, more meaningful than straightforward ELISA determination of anti-PA IgG titres. In a previous study where microsphere-associated PA was delivered intra-muscularly [11] it was shown that an ELISA titre of 10^3 (corresponding to an anti-PA IgG titre of approximately 70 μg/ml) would protect immunised animals against 10^3 MLD of STI spores in an intra-peritoneal challenge. Inclusion of rPA as a control and at an amount that has been shown to confer protection when administered by a parenteral route [10] allowed us to correlate our ELISA titres and TNA assays and we can define a TNA titre of approx 10^3 as being protective to a challenge dose of 10^3 MLD of STI spores (confirmed from challenge experiments performed in this study). It is feasible though that TNA titres as low as 10^2 will yield some level of protection and indeed Hering et al. [24] had shown positive human anti-AVA sera could have TNA titres ranging from 10^2 to 10^3.

In this work we were unable to express full length PA on the spore coat but succeeded in displaying domains 1b-3 and domain 4. The carboxy-terminal, domain 4, in particular, has been shown to contain the dominant epitopes of PA and is involved in binding to the host cell receptor [10]. Interestingly though, secretion of PA appears to enhance responses to PA domains displayed on the spore surface or conversely, display of these domains on the spore surface enhances responses to the PA secreted from the vegetative cell. This can be seen in the TNA titres of DL507, DL509 and DL511-immunized mice. Isotype analysis revealed different profiles for mice receiving rPA versus recombinant spores. In the case of rPA IgG1 levels were highest followed by IgG2b which is indicative of a Th2 bias and would be expected for responses against soluble antigen and leading to B cell activation and a humoral response. As has been noted before immunisation of full length or truncated versions of rPA in the presence of the adjuvant Alhydrogel produces a noticeable Th2 bias [10,37]. A Th2 bias is also produced when rPA is given as microspheric formulations [11]. On the other hand, in serum from animals receiving recombinant spores, IgG1 predominated followed by IgG2a instead of IgG2b. This also agrees with other studies using *B. subtilis* spores for spore coat delivery of tetanus antigens [12]. While this might appear as only a subtle difference it does imply that use of the spore is introducing a Th1 bias indicated by the higher IgG2a levels. Spores of *B. subtilis* have been shown to survive uptake by phagocytic cells and can actually germinate within the phagolysosome and undertake at least a short phase of gene expression in the newly formed vegetative cell [16]. While this fate resembles that of *B. anthracis* where the *pagA* gene is expressed within minutes of phagocytosis [38,39], the *B. subtilis* cell is of course destroyed.

For mice immunised with DL346 spores anti-PA IgG responses can only be generated if the spore germinates, the vegetative cell emerges from its protein shell and metabolism is restored. Experimentally, using RT-PCR, this process has been shown to occur within 15 min [38] but would still require the appropriate conditions of 'activation' and germination signals [40]. Although studies have suggested that *B. anthracis* can germinate in the peritoneum of the guinea pig (cited in [38]) the most likely scenario is of germination within the phagolysosome. As mentioned above *B. subtilis* spores have been shown to germinate within the phagolysosome and can initiate vegetative gene expression so we would expect PA to be synthesised and secreted within the phagolysosome mimicking the *B. anthracis* life cycle.

In conclusion, these studies have demonstrated the validity of *B. subtilis* to serve as a recombinant anthrax vaccine. In the immediate term, *B. subtilis* could, based on the preliminary studies shown here, be considered for development as a replacement of the current anthrax vaccine used in animals and composed of the spores of the Sterne strain and which is recognised as being far from ideal [1].

Acknowledgments

SMC was supported by the BBSRC (111/S19599). We gratefully acknowledge the technical assistance provided by Maggie Stokes and Tony Stagg.

References

[1] Mock M, Fouet A. Anthrax. Annu Rev Microbiol 2001;55:647–71.
[2] Baillie L. The development of new vaccines against *Bacillus anthracis*. J Appl Microbiol 2001;91:609–13.
[3] McBride BW, Mogg A, Telfer JL, Lever MS, Miller J, Turnbull PC, et al. Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. Vaccine 1998;16(8):810–7.

[4] Leppla SH, Robbins JB, Schneerson R, Shiloach J. Development of an improved vaccine for anthrax. J Clin Invest 2002;110:141–4.
[5] Muniz AE. Lymphocytic vasculitis associated with the anthrax vaccine: case report and review of anthrax vaccination. J Emerg Med 2003;25(3):271–6.
[6] Brey RN. Molecular basis for improved anthrax vaccines. Adv Drug Deliv Rev 2005;57(9):1266–92.
[7] Green BD, Battisti L, Koehler TM, Thorne CB, Ivins BE. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect Immun 1985;49(2):291–7.
[8] Klimpel KR, Molloy SS, Thomas G, Leppla SH. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc Natl Acad Sci USA 1992;89(21):10277–81.
[9] Petosa C, Collier RJ, Klimpel KR, Leppla SH, Liddington RC. Crystal structure of the anthrax toxin protective antigen. Nature 1997;385(6619):833–8.
[10] Flick-Smith HC, Walker NJ, Gibson P, Bullifent H, Hayward S, Miller J, et al. A recombinant carboxy-terminal domain of the protective antigen of *Bacillus anthracis* protects mice against anthrax infection. Infect Immun 2002;70(3):1653–6.
[11] Flick-Smith HC, Eyles JE, Hebdon R, Waters EL, Beedham RJ, Stagg TJ, et al. Mucosal or parenteral administration of microsphere-associated *Bacillus anthracis* protective antigen protects against anthrax infection in mice. Infect Immun 2002;70(4):2022–8.
[12] Duc LH, Hong HA, Fairweather N, Ricca E, Cutting SM. Bacterial spores as vaccine vehicles. Infect Immun 2003;71:2810–8.
[13] Casula G, Cutting SM. *Bacillus* probiotics: spore germination in the gastrointestinal tract. Appl Environ Microbiol 2002;68:2344–52.
[14] Duc LH, Hong HA, Cutting SM. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen presentation. Vaccine 2003;21:4215–24.
[15] Rhee KJ, Sethupathi P, Driks A, Lanning DK, Knight KL. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J Immunol 2004;172: 1118–24.
[16] Duc LH, Hong HA, Uyen NQ, Cutting SM. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine 2004;22:1873–85.
[17] Hong HA, Duc LH, Cutting SM. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 2005;29:813–35.
[18] Beedham RJ, Turnbull PC, Williamson ED. Passive transfer of protection against *Bacillus anthracis* infection in a murine model. Vaccine 2001;19(31):4409–16.
[19] Youngman P, Perkins J, Losick R. Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid 1984;12:1–9.
[20] Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. 3rd ed Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.
[21] Harwood CR, Cutting SM. Molecular Biological Methods for Bacillus. Chichester, England: John Wiley & Sons Ltd.; 1990.
[22] Guerout-Fleury AM, Frandsen N, Stragier P. Plasmids for ectopic integration in *Bacillus subtilis*. Gene 1996;180(1–2):57–61.
[23] Nicholson WL, Setlow P. Sporulation, germination and outgrowth. In: Harwood CR, Cutting SM, editors. Molecular Biological Methods for Bacillus. Chichester, UK: John Wiley & Sons Ltd.; 1990. p. 391–450.
[24] Hering D, Thompson W, Hewetson J, Little S, Norris S, Pace-Templeton J. Validation of the anthrax lethal toxin neutralization assay. Biologicals 2004;32(1):17–27.
[25] Brossier F, Levy M, Mock M. Anthrax spores make an essential contribution to vaccine efficacy. Infect Immun 2002;70:661–4.
[26] Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, Oggioni MR, et al. Surface display of recombinant proteins on *Bacillus subtilis* spores. J Bacteriol 2001;183:6294–301.
[27] Mauriello EMF, Duc LH, Isticato R, Cangiano G, Hong HA, De Felice M, et al. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. Vaccine 2004;22:1177–87.
[28] Welkos SL, Keener TJ, Gibbs PH. Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect Immun 1986;51(3):795–800.
[29] Flick-Smith HC, Waters EL, Walker NJ, Miller J, Stagg AJ, Green M, et al. Mouse model characterisation for anthrax vaccine development: comparison of one inbred and one outbred mouse strain. Microb Pathog 2005;38(1):33–40.
[30] Williamson ED, Hodgson I, Walker NJ, Topping AW, Duchars MG, Mott JM, et al. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect Immun 2005;73(9):5978–87.
[31] Ivins BE, Welkos SL, Knudson GB, Little SF. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect Immun 1990;58(2):303–8.
[32] Ivins BE, Welkos SL. Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. Infect Immun 1986;54(2):537–42.
[33] VanCott JL, Staats HF, Pascual DW, Roberts M, Chatfield SN, Yamamoto A, et al. Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunisation with live recombinant *Salmonella*. J Immunol 1996;156:1504–14.
[34] Isaka M, Yasuda Y, Mizokami M, Kozuka S, Taniguchi T, Matano K, et al. Mucosal immunization against hepatitis B virus by intranasal coadministration of recombinant hepatitis B surface antigen and recombinant cholera toxin B subunit as an adjuvant. Vaccine 2001;19:1460–6.
[35] Roberts M, Li J, Bacon A, Chatfield S. Oral vaccination against tetanus: comparison of the immunogenicities of *Salmonella* strains expressing fragment C from the *nirA* and *htrA* promoters. Infect Immun 1998;66:3080–7.
[36] Garmory HS, Titball RW, Griffin KF, Hahn U, Bohm R, Beyer W. *Salmonella enterica* serovar *typhimurium* expressing a chromosomally integrated copy of the *Bacillus anthracis* protective antigen gene protects mice against an anthrax spore challenge. Infect Immun 2003;71(7):3831–6.
[37] Williamson ED, Beedham RJ, Bennett AM, Perkins SD, Miller J, Baillie LW. Presentation of protective antigen to the mouse immune system: immune sequelae. J Appl Microbiol 1999;87(2):315–7.
[38] Cote CK, Rossi CA, Kang AS, Morrow PR, Lee JS, Welkos SL. The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb Pathog 2005;38(5–6):209–25.
[39] Guidi-Rontani C, Weber-Levy M, Labruyere E, Mock M. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol Microbiol 1999;31:9–17.
[40] Moir A, Smith DA. The genetics of bacterial spore germination. Annu Rev Microbiol 1990;44:531–53.