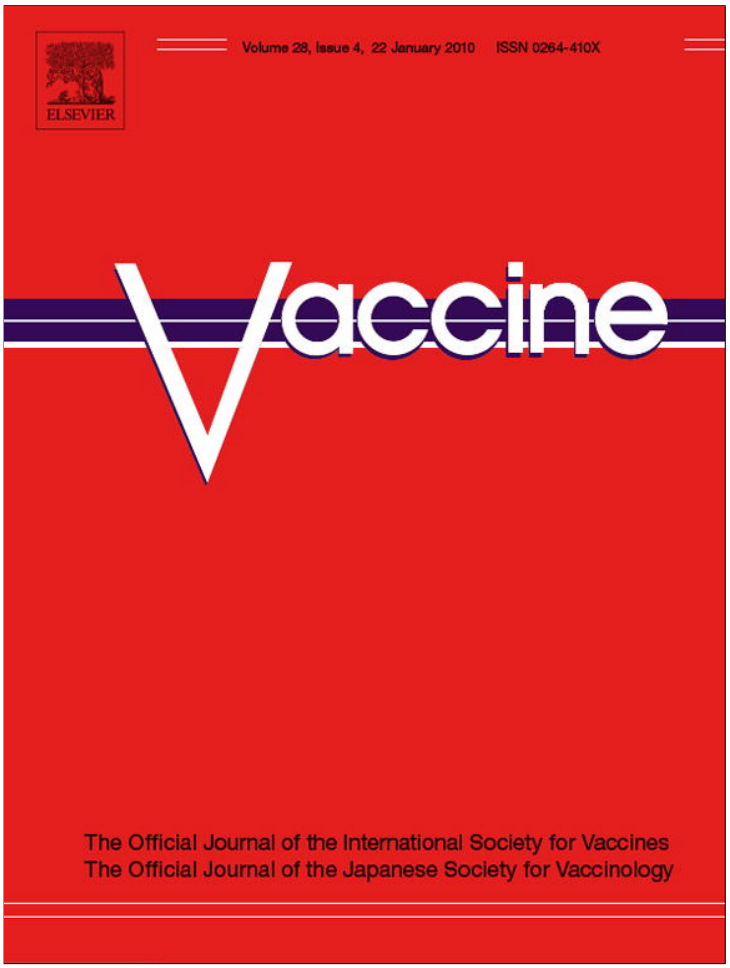

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Mucosal delivery of antigens using adsorption to bacterial spores

Jen-Min Huang[a,1], Huynh A. Hong[a,1], Hoang Van Tong[b], Tran H. Hoang[a], Alain Brisson[c], Simon M. Cutting[a,*]

[a] School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK
[b] Department of Microbiology, Vietnam Military Medical University Ha Dong, Ha Noi, Viet Nam
[c] Imagerie Moleculaire et NanoBioTechnologie-IECB, UMR-CNRS 5248 Chimie et Biologie des Membranes et Nanoobjets, Batiment B8, Avenue des Facultes, F-33405 Talence Cedex, France

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ABSTRACT

The development of new-generation vaccines has followed a number of strategic avenues including the use of live recombinant bacteria. Of these, the use of genetically engineered bacterial spores has been shown to offer promise as both a mucosal as well as a heat-stable vaccine delivery system. Spores of the genus *Bacillus* are currently in widespread use as probiotics enabling a case to be made for their safety. In this work we have discovered that the negatively charged and hydrophobic surface layer of spores provides a suitable platform for adsorption of protein antigens. Binding can be promoted under conditions of low pH and requires a potent combination of electrostatic and hydrophobic interactions between spore and immunogen. Using appropriately adsorbed spores we have shown that mice immunised mucosally can be protected against challenge with tetanus toxin, *Clostridium perfringens* alpha toxin and could survive challenge with anthrax toxin. In some cases protection is actually greater than using a recombinant vaccine. Remarkably, killed or inactivated spores appear equally effective as live spores. The spore appears to present a bound antigen in its native conformation promoting a cellular (T_h1-biased) response coupled with a strong antibody response. Spores then, should be considered as mucosal adjuvants, most similar to particulate adjuvants, by enhancing responses against soluble antigens. The broad spectrum of immune responses elicited coupled with the attendant benefits of safety suggest that spore adsorption could be appropriate for improving the immunogenicity of some vaccines as well as the delivery of biotherapeutic molecules.

1. Introduction

The use of live bacteria as vaccine delivery systems has provided one arm in the push to develop new and more effective vaccines. Live bacterial vaccines include a number of species including those of *Salmonella*, *Shigella*, *Escherichia coli*, *Lactobacillus* and *Bacillus* [1]. In some cases the strategy used is to exploit the life cycle of a pathogen, for example, one oral dose of recombinant, attenuated, *Salmonella enterica* serovar Typhi Ty21a is sufficient to generate a potent immune response because the bacterium efficiently targets the gut-associated lymphoid tissue (GALT) [2]. In the case of *Bacillus* which is the focus of this paper, *Bacillus subtilis* engineered to express heterologous antigens on the surface of the spore or within the germinating spore can be used for oral or nasal delivery of antigens and confer protective immunity. With *B. subtilis* the spore, as a dormant life form, has further advantages of being heat-stable, non-pathogenic and in current use in humans and animals as a probiotic [3]. Recombinant spores expressing protective antigens from *Clostridium tetani* [4], *Clostridium perfringens* [5], *Bacillus anthracis* [6], as well as the parasite *Clonorchis sinensis* [7] have all been shown to confer protection using animal models. With the exception of *B. anthracis*, a mucosal route of delivery has been used in every case demonstrating that recombinant spores could be utilized in a simplified vaccination strategy. On the other hand the use of recombinant bacteria also raises concerns over the use of genetically modified microorganisms and clearance of the bacterium from the host following delivery [1].

A second goal in the development of better vaccines is in identifying new adjuvants that can boost immunogenicity of otherwise, weakly immunogenic antigens (e.g., recombinant protein subunits and synthetic peptides). Until recently only one adjuvant, alum, has been licensed for human use and a number of potential vaccine adjuvants are under development including immunostimulatory adjuvants, mucosal adjuvants, lipid particles and particulate adjuvants [8,9]. Each of these have their strengths but also weaknesses. One of these new classes of adjuvants, referred to as particulate adjuvants (e.g., liposomes, virosomes, virus-like particles,

* Corresponding author. Tel.: +44 01784 443760; fax: +44 01784 414224.
E-mail address: s.cutting@rhul.ac.uk (S.M. Cutting).
[1] These authors contributed equally.

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poly-lactide-co-glycolide (PLG) microspheres and immune stimulating complexes (ISCOMS)), are particularly encouraging because they mimic the pathogens the immune system has evolved to destroy. Particulate adjuvants efficiently target antigen presenting cells (APCs) and once internalised within the cell are processed by the class I and class II MHC (major histocompatibility complex) pathways leading to antigen presentation on the surface of the APC. Biodegradable PLGs have been shown to induce CTL responses a prerequisite for combating intracellular pathogens [10]. Studies on copolymer adjuvants have shown that for induction of a broad range of immune responses (antibody and cell-mediated) it is necessary that the antigen remains in its native form which can be achieved if bound to the surface of a suitable surface [11]. Many antigens prepared in water-in-oil emulsions are rapidly engulfed by APCs, degraded, and then enter the class II pathway leading to antibody production but in a challenge model, fail to protect. This is thought to be due to the failure to produce the IgG2a isotype that is important for recognition of conformational epitopes [11]. Stabilisation of antigens on inert surfaces in their native form coupled with the ability to induce potent immune responses remain one of the challenges in vaccine and adjuvant formulations.

Previous studies have shown that *Bacillus* spores possess adjuvant properties [12,13] and in this work we show that this is brought about by binding of the antigen to the spore surface. Spores then, serve as antigen carriers, closely resembling the properties of microparticulate adjuvants. Using this approach, we demonstrate the utility of antigen-adsorbed spores in conferring protective immunity to three bacterial pathogens following delivery by a mucosal route.

2. Materials and methods

2.1. Strains

B. subtilis strain PY79, is a standard prototrophic laboratory strain and isogenic to the 168 type strain [14]. HU58 is a non-domesticated isolate of *B. subtilis* [15]. HT251 (*amyE::cotB-GST-Cpa*$_{247–370}$) is isogenic to strain PY79 and carries a recombinant gene on its genome that expresses a modified spore coat protein, CotB, that has been fused to GST-Cpa$_{247–370}$ [5]. RH103 (*amyE::cotB-tetC*) expresses the immunogen, TTFC (tetanus toxin fragment C) from *C. tetani* on the spore surface as a chimera fused to the CotB protein [16].

2.2. Preparation of spores and general methods

Spores used in all experiments were prepared by growth and sporulation in Difco Sporulation Medium (DSM) as described elsewhere [17]. Each batch of spores was heat-treated (68 °C, 45 min) to ensure killing of all vegetative cells. Spores were suspended in sterile PBS (pH 7.4) and stored in aliquots (1×10^{11} spores/ml) at −70 °C until use. Spore counts were determined by (i) direct counting using a haemocytometer and phase-contrast microscopy, and (ii) by serial dilution and plate-counting. Extraction and analysis of spore coat proteins using SDS-PAGE was as described [17].

2.3. Zeta potential measurements

Zeta potentials of the PY79 spore isolates were measured at 24 °C with a 3000HS Malvern Zeta-sizer (Malvern Instruments Ltd., UK). Aliquots of 30 μl of spores suspended in Milli-Q water at a density of 5×10^9 spores/ml were added to 3 ml solutions of defined pH and ionic strength, as described in the text. The pH was adjusted using HCl or NaOH. The mean of two separate measurements from the same sample was determined. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation [18].

2.4. Spore adhesion to hydrocarbon (SATH) assay

The surface hydrophobicity of the PY79 spore isolates was determined using the SATH assay using *n*-hexadecane as hydrocarbon [19]. Purified spores were washed in either Milli-Q water or 1 M NaCl in Milli-Q water by centrifugation at 16,000 × *g* for 10 min and resuspended in 0.1 M NaCl at a density of 1×10^8 spores/ml. Spore suspensions (2 ml) were added to 1 ml *n*-hexadecane (Aldrich) and vortexed for 1 min, incubated at 37 °C for 10 min, and vortexed again for 30 s. The absorbance of the aqueous phase was measured at 600 nm. The mean of two measurements was determined. The percent hydrophobicity (*%H*) was determined from the absorbance of the original spore suspension (A_i) and the absorbance of the aqueous phase after incubation with hexadecane (A_f) according to the following equation: $\%H = [(A_i - A_f)/A_i] \times 100$.

2.5. Recombinant proteins

Proteins were expressed from recombinant plasmids in the *E. coli* strain BL21. With the exception of GST-Cpa$_{247–370}$, the expressed protein carried a poly-histidine tag at its 3′-end and following expression was purified using an AKTA chromatography system (Pharmacia).

(i) TTFC: pET-28b-TTFC expressed *C. tetani* TTFC as a 52.6 kDa polypeptide and has been described elsewhere [4].
(ii) PA: pET-28b-PA expressed the 83.5 kDa protective antigen (PA) from *B. anthracis*. As described elsewhere, in this cassette the secretory signal sequence permitting membrane secretion was deleted [20].
(iii) GST-Cpa$_{247–370}$: The 41 kDa hybrid protein of Sj26GST fused to the carboxy-terminal domain of *C. perfringens* alpha toxin (Cpa$_{247–370}$) has been described elsewhere [21] and was purified using a GST-binding column.
(iv) GST: pET-28b-GST expressed the 26.3 kDa glutathione S-transferase (Sj26GST) from *Shistosomas japonica* [22]. Henceforth Sj26GST is referred to as GST. The *sj26GST* gene was PCR-amplified and cloned from the vector pGEX3X13 (Pharmacia).

2.6. Antibodies

Polyclonal antibodies were raised in mice immunised by the intra-peritoneal (i.p.) route with 2 μg of purified protein on days 1, 14 and 28. Dilutions used were 1:1000 for anti-PA and 1:2000 for anti-TTFC and anti-GST-Cpa$_{247–370}$.

2.7. Binding assays

Unless indicated otherwise the general method for adsorbing proteins to spores was as follows. Suspensions containing 2×10^9 spores were centrifuged and resuspended in 0.2 ml of PBS at pH 4, pH 7 or pH 10. Unless indicated otherwise all reactions were performed in PBS at 0.15 M. Purified recombinant proteins were added to the spore suspension and the binding mixture incubated at room temperature (RT). Spores were centrifuged (1 min, RT) and the pellet washed two times with PBS buffer of the same pH as that used in the binding mixture. The washed pellet was resuspended in 100 μl of spore coat extraction buffer [17], incubated at 68 °C for 1 h to solubilise spore coat proteins and one tenth loaded onto a 12% SDS-PAGE gel.

2.8. Animals

Animals used in this work were pathogen-free, Balb/c mice (Harlan, UK) for antibody production, and for analysis of immune responses with PA or GST-$Cpa_{247-370}$. For TTFC immunity studies C57BL/6 mice (Charles River) were used. In all cases females, aged 6–8 weeks were used. Animals were housed in the Royal Holloway, University of London's animal house and work described in this paper was performed under the Home Office project licence PPL 70/6126.

2.9. Immunisations

Mice were dosed by the intra-nasal route (light anesthesia, 20 μl using a pipette tip) or intra-gastrically (oral; 0.2 ml by gavage). A naïve group of un-immunised animals was included in all experiments.

(i) TTFC studies: Groups of mice (3–10 as specified) were immunised nasally with (i) purified TTFC protein, (ii) a pre-adsorbed (PBS, pH 4) mixture of PY79 spores (2×10^9) and TTFC protein (1 μg), or (iii) a pre-adsorbed (PBS, pH 4) mixture of PY79 spores (2×10^9) and TTFC protein (5 μg) that had been washed two times with PBS buffer (pH 4). A further group consisted of autoclaved PY79 spores (2×10^9; 15 p.s.i., 30 min) that had been pre-adsorbed (PBS, pH 4) with TTFC (1 μg). Control groups included naïve (PBS, pH 4) and a group receiving PY79 (2×10^9) spores by the intra-nasal route. The dosing regime consisted of immunisations on days 1, 15, 29 and 43. One additional group of mice was given 2×10^{10} RH103 spores (in PBS, pH 7.4) orally on days 1, 2, 3, 22, 23, 24, 43, 44 and 45 as a positive control. Mice were challenged with *C. tetani* tetanus toxin on day 64.

(ii) GST-$Cpa_{247-370}$ studies: Groups of six mice were immunised by either the intra-nasal or oral routes on days 1, 21 and 42 after which the challenge was performed on day 63. For oral immunisations groups included animals receiving PY79 spores (5×10^{10}), GST-$Cpa_{247-370}$ protein (3.6 μg) and a further group receiving a mixture of PY79 spores (5×10^{10}) that had been pre-adsorbed (80 min RT; PBS pH 7.4) with 3.6 μg of GST-$Cpa_{247-370}$. A positive control consisted of 5×10^{10} spores of HT251 (*amyE::cotB-GST-*$Cpa_{247-370}$). For intra-nasal dosing one group received PY79 spores (2×10^9), another group GST-$Cpa_{247-370}$ protein (0.15 μg) and a further group a pre-adsorbed (80 min RT; PBS pH 7.4) mixture of PY79 spores (2×10^9) and GST-$Cpa_{247-370}$ protein (0.15 μg).

(iii) PA studies: Groups of six mice were dosed intra-nasally on days 1, 22 and 43. Groups received HU58 spores (2×10^9) or the same quantity of HU58 spores that had been adsorbed with 2 μg of PA protein for 1 h in PBS (pH 4).

2.10. Humoral responses

For analysis of anti-GST-$Cpa_{247-370}$ responses serum, saliva and faecal pellets were taken on days −1, 20, 40 and 60. For PA-specific responses, serum samples were taken on days −1, 21, 42 and 63 and for TTFC-responses terminal bleeds were used for IgG analysis. Lung washes for extraction of sIgA was done on lungs taken from sacrificed animals performed as described [23]. Determination of antibody titres by ELISA was as described elsewhere [4,5,24].

2.11. IFN-γ ELISA

Spleen cells (5×10^5) taken from mice were dosed with immunogen (protein, spores or protein + spores), seeded in 96-well cell culture plates in complete medium, were stimulated with 5 μg/ml of either TTFC or PA protein, 1 μg/ml ConA was used as a positive control or with medium only and cells were incubated at 37 °C in 5% CO_2. Supernatants were harvested from cultures after 6 days and analysed for IFN-γ as described [24]. IFN-γ (BD Biosciences) was used as a standard.

2.12. Toxin neutralising assays (TNA)

Measurement of anthrax toxin neutralising activity the TNA was done as described previously using a RAW264.7 cell-line [24].

2.13. Toxin challenges

Challenge of immunised mice with *C. tetani* tetanus toxin or *C. perfringens* alpha toxin were performed as described previously [4,5]. Note that challenge experiments were conducted in experiments independent from studies in which immune responses were evaluated.

2.14. Statistics

The Student's *t*-test was used to compare between groups. A *P*-value of >0.05 was considered non-significant.

2.15. Spore dimensions

Measurements of length and width of over 100 ellipsoidal spores were taken from transmission electron microscopic analysis of embedded spores. Surface volume and area were determined as described elsewhere [40].

3. Results

3.1. Oral vaccination of mice with spores pre-adsorbed with an immunogen protects mice against challenge with C. perfringens alpha toxin

In a previous study, a recombinant *B. subtilis* strain, HT251, was engineered to express GST-$Cpa_{247-370}$ on the spore surface fused to the outer spore coat protein CotB [5]. $Cpa_{247-370}$ is the C-terminal fragment of *C. perfringens* alpha toxin (Cpa) and when fused to GST has been shown to confer protective immunity [21]. Mice dosed orally or nasally with three doses of HT251 spores were protected against a 12 LD_{50} challenge dose of alpha toxin [5]. Although not previously published we report here an intriguing observation from experiments conducted in parallel. Using the same three-dose regime as with recombinant HT251 spores, mice were administered a mixture of GST-$Cpa_{247-370}$ protein and non-recombinant PY79 spores. For the oral route, for each dose, 3.6 μg of GST-$Cpa_{247-370}$ was mixed with PBS buffer (pH 7.4) corresponding to the amount of recombinant GST-$Cpa_{247-370}$ expressed in one single dose of HT251 spores. For the nasal route 0.15 μg was used. Our analysis of GST-$Cpa_{247-370}$-specific IgG titres following oral and intra-nasal dosing revealed that the levels and kinetics of antibody responses were indistinguishable from recombinant HT251 spores (Fig. 1A and B). By contrast, PY79 spores and protein alone produced no significant levels ($P > 0.05$) of GST-$Cpa_{247-370}$-specific responses. We evaluated the ability of mice immunised with spore-immunogen mixtures to survive challenge with alpha toxin (Table 1). Protection was obtained in nasally dosed mice to a 6 LD_{50} dose of toxin while for oral dosing one mouse was able to survive the same dose of toxin (6 LD_{50}). Since neither spores nor protein alone could induce antibody responses the most straightforward explanation is that spores associate with the administered antigen facilitating their interaction and uptake by immune cells.

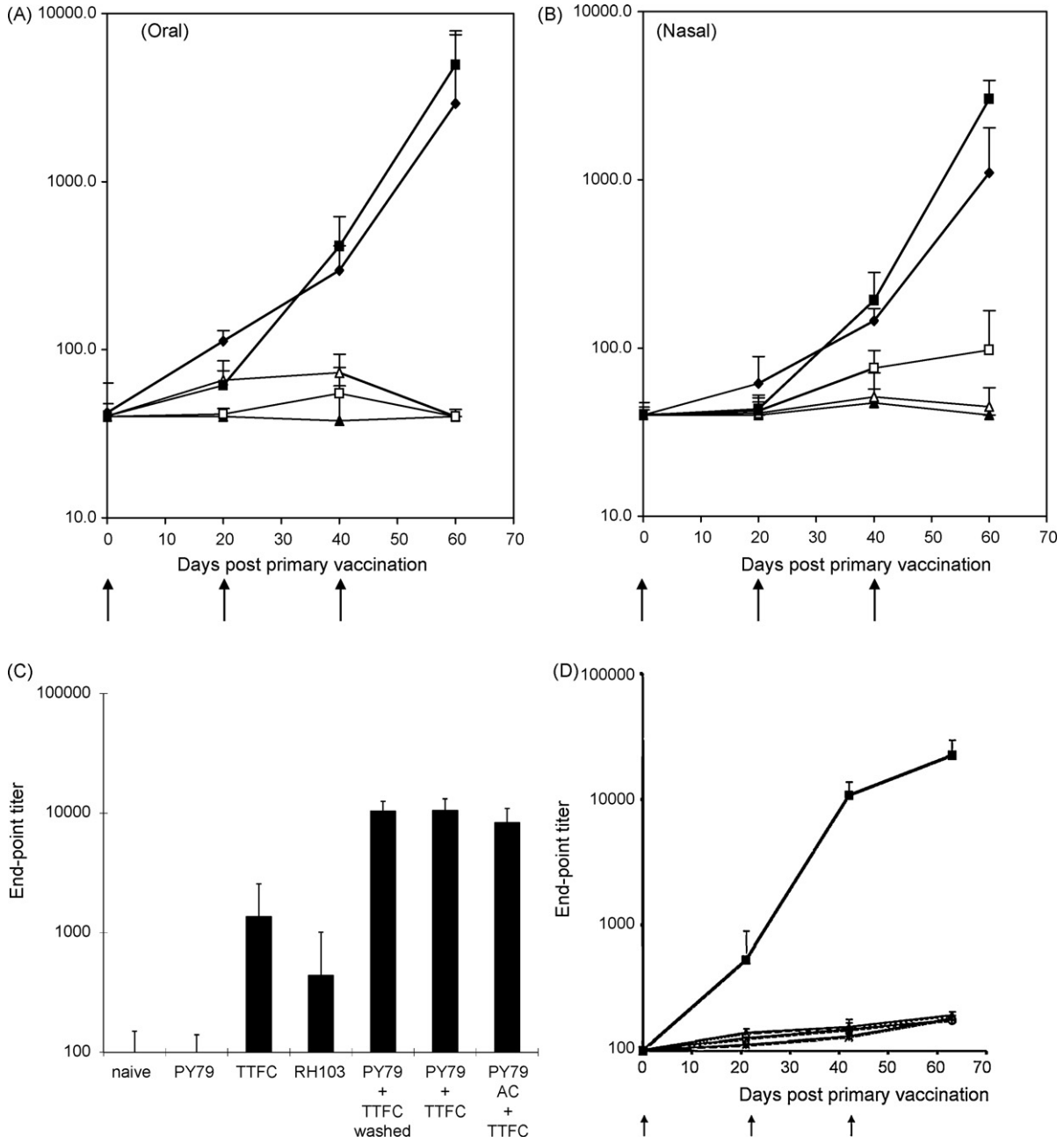

Fig. 1. Antigen-specific IgG responses. Antigen-specific IgG was measured in serum from individual mice by ELISA. (Panels A and B) GST-Cpa$_{247–370}$-specific IgG responses after oral (A) or intranasal (B) immunisation. Mice were immunised (↑) with spores of strain HT251 (*cotB-GST-Cpa*$_{247–370}$; ■), PY79 (non-recombinant; △), a pre-adsorbed mixture of PY79 spores and GST-Cpa$_{247–370}$ polypeptide (♦; 3.6 μg/dose for the oral route and 0.15 μg/dose for the nasal) and pure GST-Cpa$_{247–370}$ protein (□; 3.6 μg/dose for the oral route and 0.15 μg/dose). (Panel C): TTFC-specific IgG responses after nasal administration of spores to mice. Serum was from terminal bleeds of animals immunised as indicated. (Panel D) PA-specific IgG responses after nasal administration (↑) of spores of strain HU58 (○), a pre-adsorbed mixture of HU58 and PA polypeptide (■; 2 μg/dose), pure PA protein (△; 2 μg/dose) to mice and a naïve group (*) received PBS only.

3.2. Adsorption of proteins to spores

To determine whether a protein immunogen could bind to spores we designed an adsorption experiment mixing the GST-Cpa$_{247–370}$ protein (mwt., 40.4 kDa; p*I*, 5.7) with PY79 spores and then analysing samples of spore coat protein extract and supernatants for the presence of GST-Cpa$_{247–370}$ by immunoblotting (Fig. 2A). Our data showed that at pH 4 and pH 7, GST-Cpa$_{247–370}$ was efficiently adsorbed onto the spore coat but with low levels of binding at pH 10. We calculated by immunoquantification that 2.6×10^{-4} pg of protein had adsorbed per spore ($\sim 3.7 \times 10^{3}$ molecules/spore).

We extended this study by determining whether other protein antigens could be adsorbed onto the spore surface. Using tetanus toxin fragment C (TTFC; mwt., 52.6 kDa; p*I*, 6.34) of *C. tetani* and the protective antigen (PA; mwt., 83.5; p*I*, 5.87) of *B. anthracis* both antigens were found to bind efficiently to spores (Fig. 2A). In both cases though, binding was only detected at pH 4 with 4.9×10^{-4} pg of TTFC bound/spore (5.4×10^{3} molecules/spore) and 4.6×10^{-4} pg of PA/spore (3.2×10^{3} molecules/spore). We measured the saturation of binding by adsorbing increasing amounts of protein to a suspension of 2×10^{9} PY79 and found that the maximum amount of TTFC or GST-Cpa$_{247–370}$ that could adsorb to spores was ~1.0 μg of TTFC or ~0.5 μg GST-Cpa$_{247–370}$/2×10^{9} spores (Supplementary

Table 1
Protection against challenge with alpha-toxin and tetanus toxin in immunised mice.

Alpha toxin challenge experiments[a]

Group	Route	Dose of alpha toxin (LD_{50})	No. survivors	Mean time to death ± (h)
Naïve	–	2	0/4	4.5 ± 0.5
PY79 spores	Oral	6	0/6	2.0 ± 0.5
3.6 μg GST-$Cpa_{247–370}$	Oral	6	0/6	2.5 ± 0.5
PY79 spores + 3.6 μg GST-$Cpa_{247–370}$	Oral	6	1/6	–
		12	0/6	–
HT251 (*cotB*-GST-$Cpa_{247–370}$)	Oral	6	6/6	–
		12	3/6	
PY79 spores	Intra-nasal	6	0/6	2.0 ± 0.5
0.15 μg GST-$Cpa_{247–370}$	Intra-nasal	6	0/6	2.5 ± 0.3
PY79 spores + 0.15 μg GST-$Cpa_{247–370}$	Intra-nasal	6	5/6	–
		12	0/6	
HT251 (*cotB*-GST-$Cpa_{247–370}$)	Intra-nasal	6	6/6	–

Tetanus toxin challenge experiments[b]

Group	Route	pH[c]	Toxin challengedose (LD_{50})	No. of survivors/total
Naïve	–	–	2	0/10
PY79 spores	Intra-nasal	7	2	0/3
			50	0/7
1 μg TTFC protein	Intra-nasal	4	50	0/9
RH103 spores (CotB-TTFC)	Oral	7	20	1/10
PY79 spores + 5 μg TTFC protein then washed[d]	Intra-nasal	4	50	8/10
PY79 spores + 1 μg TTFC protein	Intra-nasal	4	50	9/10
Autoclaved PY79 spores + 1 μg TTFC protein	Intra-nasal	4	50	5/10

[a] Mice were immunised with PY79 spores, GST-$Cpa_{247–370}$ protein or PY79 spores adsorbed with GST-$Cpa_{247–370}$ protein by the oral or intra-nasal routes using dosings on days 1, 21 and 42. A challenge on immunised animals using alpha toxin was conducted 63 days after the start of the dosing regime. A control group received recombinant HT251 spores that expressed GST-$Cpa_{247–370}$ on the spore coat as a fusion to the coat protein CotB.
[b] Groups of mice immunised were immunised with PY79 spores that had been pre-adsorbed with recombinant TTFC protein. Variations included the use of autoclaved spores or pre-adsorption followed by washing of the spore. After four intra-nasal doses on day 63 animals were challenged (sub-cutaneously) with tetanus toxin. Individuals showing no symptoms after 14 days were considered immune. A control group consisted on mice receiving nine doses of RH103 spores by the intra-gastric route.
[c] pH of the PBS with which spores or protein was administered to animals.
[d] Spores and protein pre-adsorbed (1 h, RT) in PBS buffer at pH 4, followed by two washes in PBS (pH 4).

Fig. 1). Electron microscopy revealed that PY79 spores had a surface area of 2.3 μm^2 (based on average length, 1.38 μm, and width, 0.63 μm, measurements) showing that approximately 1.6×10^{15} protein binding sites per square meter (~5% of surface area) are present at the spore surface.

Since GST-$Cpa_{247–370}$ was a chimera of GST and Cpa we wondered whether the binding observed at pH 7 and pH 10 was due to one of the component polypeptides. Cpa was unstable without fusion to GST so we examined binding of GST alone (Fig. 2A). GST was found to bind at all three pH values and while we cannot be certain it is possible that GST confers upon the GST-$Cpa_{247–370}$ polypeptide the ability to bind at all three pH conditions. Surprisingly, each of the three proteins studied here could adsorb to autoclaved PY79 spores as efficiently as 'live' spores (Fig. 2B shows binding of TTFC). Since spores adsorbed with GST-$Cpa_{247–370}$ were able to generate immune responses following oral administration we asked whether the spore conferred protection to simulated gastric juices containing pepsin as described elsewhere [25]. Under these conditions we failed to detect any protection conferred by the spore to the adsorbed protein that was degraded (data not shown). Since the outermost layer of the spore coat of *B. subtilis* is comprised exclusively of five coat proteins, CotA, CotB, CotC, CotG and CotF we examined adsorption of proteins onto isogenic null mutants lacking each of the Cot genes. In each case we were able to show that in each case protein adsorption was less than in wild type PY79 spores but never abolished (data not shown). We infer then that adsorption is most multivalent and non-specific.

The stability of binding was examined by measuring the dissociation of bound immunogen (TTFC) from adsorbed spores (Fig. 2C). Our results showed that TTFC bound to spores at low pH was stable for at least 90 min with no significant dissociation. When suspended in buffers of higher pH only a small amount of protein was detectable in the supernatant demonstrating that protein adsorption to spores was stable. All three recombinant proteins were shown to bind rapidly to spores and maximum levels of binding being achieved in approximately 10 min (Fig. 3).

3.3. Surface hydrophobicity and surface charge properties of PY79 spores

The surface hydrophobicity of PY79 spores was characterized by the SATH assay [19] and electro-kinetic properties were characterized by zeta potential measurements which are an indicator or surface charge [26]. Using the SATH assay PY79 spores presented an almost constant hydrophobicity, close to 95%, over the pH range of 3–8 (Fig. 4). The zeta potentials of spores were determined between pH 1 and 8 in water (Fig. 4) with negative zeta potentials presented over the entire pH range. Remarkably, the zeta potential presented a significant peak broadening at pH 1, indicating that this pH is close to the isoelectric point. In complementary experiments, zeta potentials were measured at pHs 3, 5 and 7 for spores suspended in the presence of either 1 M NaCl or 5 mM $CaCl_2$. The zeta potentials were higher than those measured in water, yet remained negative (data not shown). The fact that zeta potentials become less negative in the presence of sodium or calcium ions is

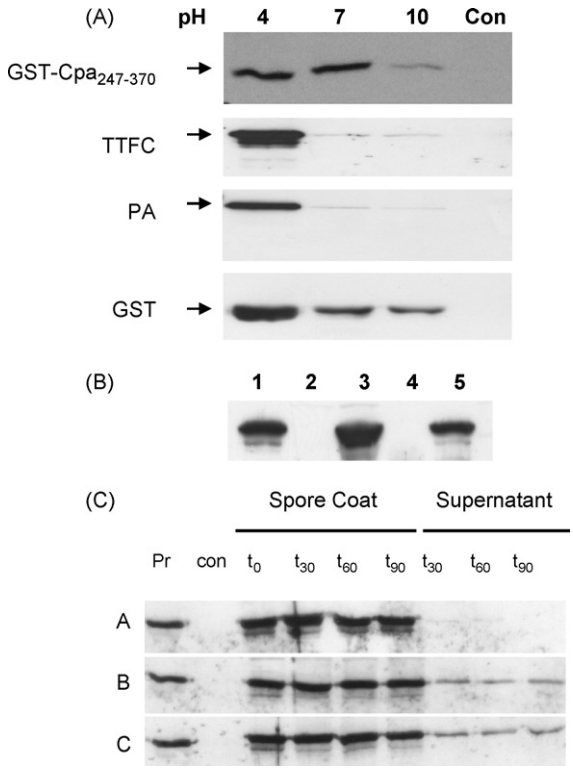

Fig. 2. Adsorption of immunogens to spores. (Panel A) Purified suspensions of PY79 spores in different pH buffers (PBS) were mixed with purified recombinant protein (2 μg) for 1 h at RT. Spores were centrifuged, washed two times and coat proteins extracted. Using one tenth of the extraction western blotting of size-fractionated proteins was used for detection. In each case one predominant band was detected which is shown in the figure. 0.1 μg of purified recombinant protein is shown for comparison. The control lane (Con) shows the corresponding blot from spores taken through the entire procedure without protein. (Panel B) Binding of 2 μg TTFC to spore coats as described in Panel A. Lane 1, 0.1 μg of TTFC as a control; lane 2, unbound PY79 spores; lane 3, PY79 spores + TTFC; lane 4, unbound autoclaved PY79 spores; lane 5, autoclaved PY79 spores + TTFC. (Panel C) Suspensions of spores (2×10^9 c.f.u.) were centrifuged and resuspended in 0.2 ml of PBS at pH 4. Purified recombinant TTFC protein (2 μg) was added to the spore suspension and the binding mixture incubated for 1 h at RT. Spores were centrifuged and the pellet washed two times with PBS at pH4. The washed pellet was next resuspended in 200 μl of PBS at either pH4 (lane A), pH 7 (lane B) or pH 10 (lane C). At indicated time points (30, 60 and 90 min), spores were centrifuged and the supernatants saved and one twentieth loaded onto a 12% SDS-PAGE gel. Pellets were resuspended in 100 μl of spore coat extraction buffer, incubated at 68 °C for 1 h to remove spore coat proteins from spores and one tenth loaded onto a 12% SDS-PAGE gel. For controls 0.1 μg of the recombinant protein was run on the gel.

due to the cation-specific screening effect exerted on spore negative charges.

3.4. Ionic and hydrophobic interactions are important for protein adsorption

With the exception of GST-$Cpa_{247-370}$ protein adsorption was greatest at pH 4 suggesting that ionic interactions must play a role in binding since this was below the protein isoelectric point. Proteins with a net positive charge would be expected to efficiently bind to PY79 spores exhibiting a net negative charge. However, we found that spores adsorbed with TTFC that had been washed with 1 M NaCl only removed approximately 30% of the bound immunogen demonstrating that binding could not be exclusively ionic in nature (Fig. 5A). Washing with Triton X-100 though, failed to release the adsorbed protein but washing with a combination of Triton X-100 and NaCl removed approximately 70% of the bound protein demonstrating hydrophobic interactions (Fig. 5A).

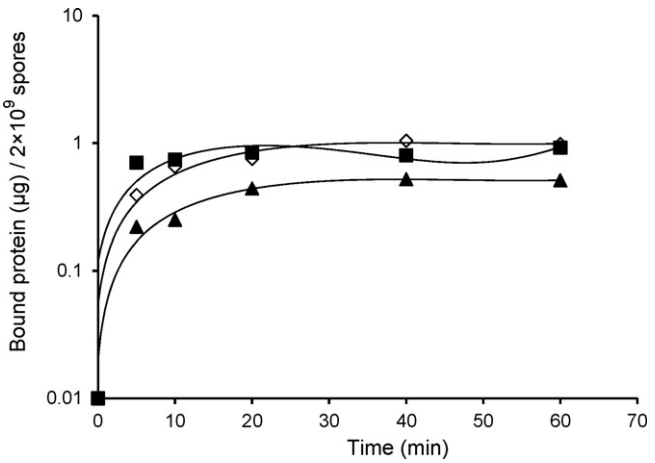

Fig. 3. Kinetics of adsorption. Suspensions of spores (2×10^9) were mixed with 10 μg of recombinant protein in PBS (pH 4) and incubated at RT and samples taken thereafter. Spores were washed two times with PBS (pH 4). ○, TTFC + PY79 spores; ▲, GST-$Cpa_{247-370}$ + PY79 spores; ■, PA + HU58 spores.

We also measured adsorption of TTFC in PBS buffers (each at pH 4) containing different concentrations of NaCl (Fig. 5B). At low salt concentrations binding was strong and sharply declined as the concentration increased to about 1 M. At NaCl concentrations above 1 M binding of TTFC steadily increased to its highest levels at 4 M NaCl. The binding studies shown in Fig. 2 were made in PBS buffer at 0.15 M (pH 4) enabling high levels of binding where charge plays the dominant role in adsorption. The increasing ability of protein to bind at higher salt concentrations indicates the importance of hydrophobic interactions [27].

The biphasic evolution of protein binding to spores is reminiscent of the well-known U-shaped curve of protein solubility versus ionic strength. At low salt concentrations, the binding of proteins to spores is driven by electrostatic interactions, the spore negative charges acting as negative counter-charges for the positive protein charges. At high salt concentration, protein solubility decreases due to a deficit in water molecules needed for solvation, which favours either the establishment of hydrophobic interactions between proteins and between proteins and spores, or the establishment of electrostatic interactions between spore negative charges and protein positive charges. The balance between electrostatic and hydrophobic interactions will obviously depend on the pH and vary between proteins.

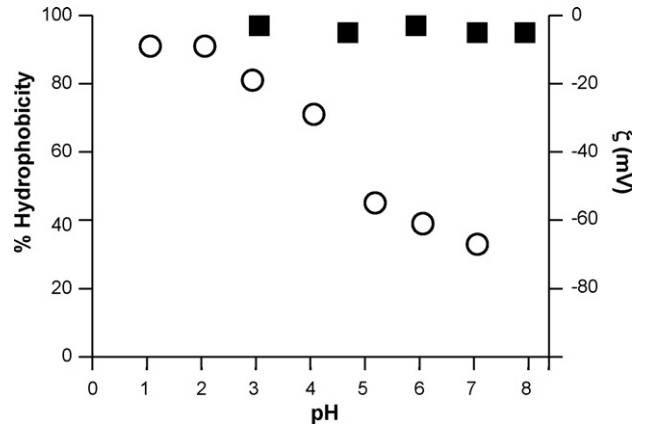

Fig. 4. Electro kinetics and hydrophobicity of *B. subtilis* PY79 spores. Zeta potential measurements of purified suspensions of PY79 spores (○) in water between pH 1 and 12. Hydrophobicity of PY79 spores (■) determined by the SATH assay in the pH range of 3–8.

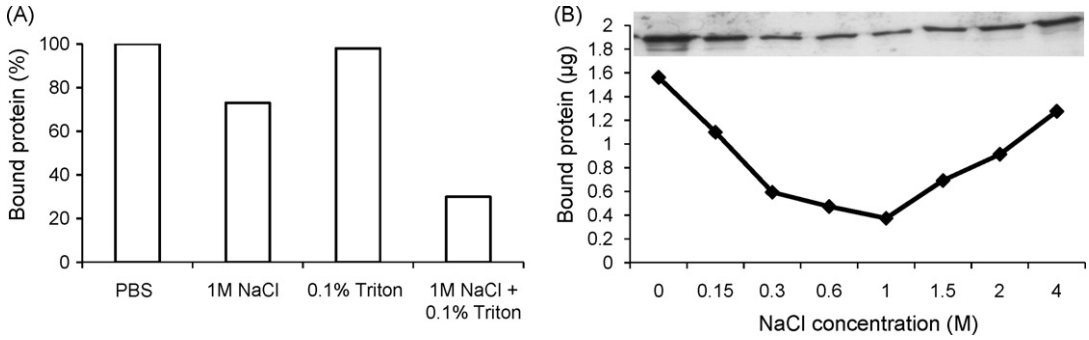

Fig. 5. Ionic and hydrophobic properties of protein adsorption. (Panel A) Recombinant TTFC (10 μg) was mixed with purified suspensions of PY79 spores (2×10^9) in 0.2 ml PBS (pH 4, 0.15 M) for 1 h at RT. After two washes with PBS, the pellet was resuspended in pH 4 PBS, 1 M NaCl, 0.1% Triton X-100, or 1 M NaCl + 0.1% Triton X-100 and incubated at RT for 15 min followed by two washes with the same buffer used for resuspension after which coat proteins were extracted. The data showed are the percentage of remaining bound protein. (Panel B) Purified recombinant TTFC (10 μg) was mixed with purified suspensions of PY79 spores (2×10^9) in 0.2 ml PBS (pH 4) containing different concentrations of NaCl (0–4 M). All the binding mixtures were incubated at RT for 1 h. Spores were centrifuged, washed two times in the PBS buffer containing the same NaCl concentration and coat proteins extracted and displayed as the total amount of protein extracted/2×10^9 spores.

3.5. Inactive spores can be used as a vaccine delivery vehicle

TTFC is the protective antigen used in evaluations of vaccines against tetanus [28]. It has been expressed on the spore coat as a chimera fused to the spore coat protein CotB where oral delivery of recombinant spores has been shown to protect against challenge with tetanus toxin [4]. We constructed a series of experiments to determine the efficiency of PY79 spores to deliver TTFC intra-nasally using a challenge experiment at the end of the dosing regime (Table 1). One group of mice was dosed four times with spores that had been mixed with an excess of TTFC protein (5 μg) at pH 4 and then washed with PBS (pH 4) to remove any unbound protein. The amount of TTFC that could bind under these conditions was calculated as approximately 1 μg/dose.

Mice dosed with these spores were protected against a tetanus toxin challenge dose of 50 LD_{50}. Other groups of mice examined were animals dosed with PY79 spores that had been mixed with 1 μg of TTFC without washing and which also showed protection against a challenge dose of 50 LD_{50}. Mice dosed with the same quantity of TTFC protein, but without spores, failed to be protected though. One further immunisation group was animals dosed with autoclaved PY79 spores that had been adsorbed with TTFC protein (1 μg). These animals showed the same level of protection to challenge with tetanus toxin (50 LD_{50}). As a control we also immunised mice by the intra-gastric route with recombinant RH103 spores (nine doses) that expressed TTFC on the spore surface. Using 2×10^{10} spores for each immunisation approximately 2 μg of TTFC would be delivered per dose and we were able to achieve protection to a 20 LD_{50} challenge dose in agreement with previous work [4]. These experiments demonstrate firstly, that spores pre-adsorbed with an antigen can be delivered by a mucosal route and could neutralise tetanus toxin *in vivo*. Second, that inactive or killed, spores are as efficacious as live spores.

3.6. Vaccination against anthrax

Recombinant *B. subtilis* spores that express the protective antigen (PA) of *B. anthracis* have been shown to protect mice against challenge with anthrax toxin when administered by a parenteral route [20]. The production of toxin neutralising antibodies was shown to be dependant upon display of PA (or fragments of PA) on the spore surface or secretion from the germinating spore [20]. In light of our studies with TTFC and GST-$Cpa_{247–370}$ we wondered then whether PA, adsorbed to spores, could be used to vaccinate mice against anthrax. In the first instance, we measured toxin neutralising antibodies (TNA) as an indicator since studies have shown that the level of neutralising antibodies correlates well with protection [20,29,30]. To determine whether the binding phenomena was specific to the laboratory PY79 strain we used a non-domesticated strain of *B. subtilis* referred to as HU58 for binding [15]. We first confirmed binding of PA to HU58 spores at pH 4, with approximately 9.34×10^{-4} pg of PA bound per spore almost double that obtained with PY79 spores (Supplementary Fig. 1). Mice dosed nasally three times with HU58 spores adsorbed with PA generated high levels of PA-specific IgG and sIgA significantly greater ($P < 0.05$) than groups dosed with HU58 spores or PA alone (Fig. 6). Neutralising antibodies (a titre of 2000) were only obtained in mice dosed with spores adsorbed with PA (Fig. 6) and were greater than the amount (1000) that has been shown to protect mice against an intra-peritoneal challenge of $>10^3$ MLD (2000 LD_{50}) of *B. anthracis* STI spores [20,29].

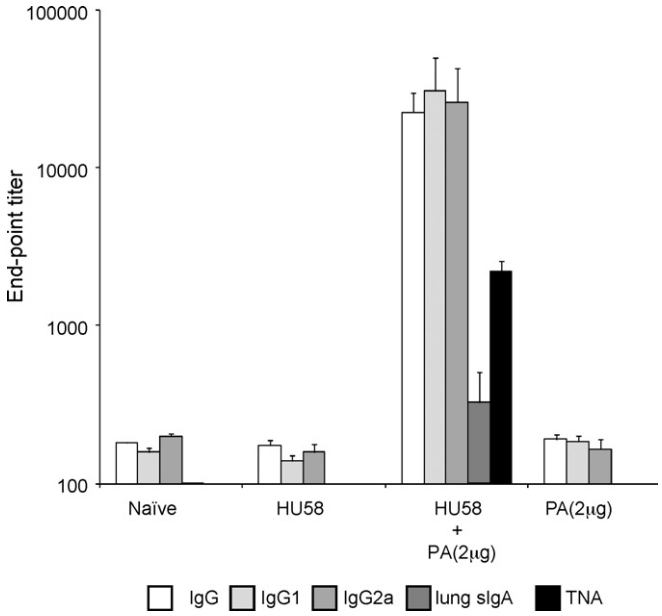

Fig. 6. PA-specific antibody responses. Mice were immunised intra-nasally with three doses of HU58 spores pre-adsorbed with PA (2 μg) and serum (IgG, IgG1, IgG2a) and mucosal antibody (sIgA) titres determined. Anthrax neutralising antibodies were determined, *in vitro*, by the toxin neutralisation assay (TNA). Controls included naïve mice, mice immunised with HU58 spores alone or with PA protein alone. Data are presented as arithmetic means and error bars are standard deviation.

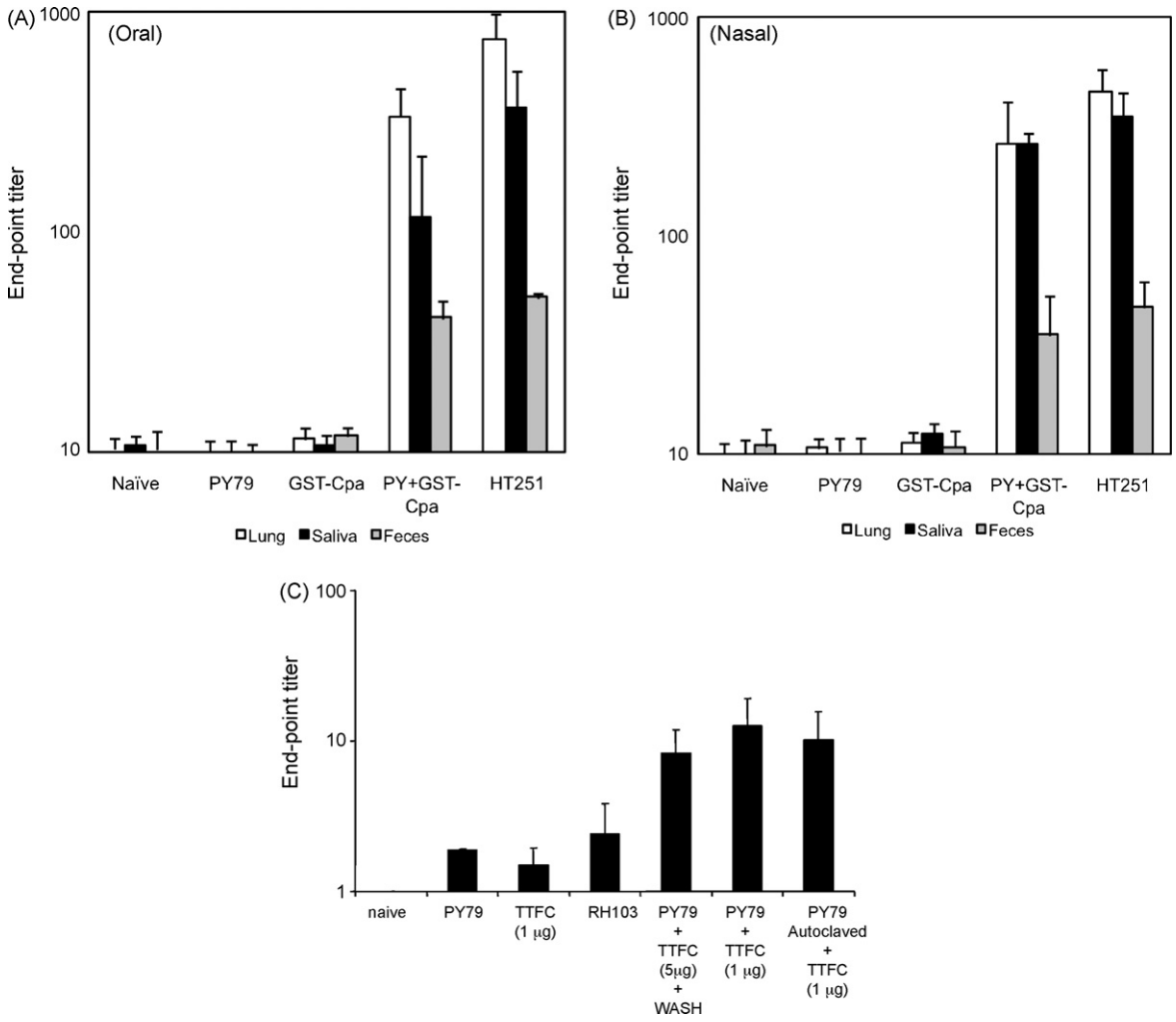

Fig. 7. Antigen-specific mucosal responses. Antigen-specific sIgA measured in lung washes, saliva and faeces from mice immunised in the immunisations experiments described in the text. Panels A and B show analysis of GST-Cpa$_{247–370}$-specific IgA taken at day 60. Panel C shows TTFC-specific sIgA obtained in lung washes taken at day 63.

3.7. Immune responses following administration of pre-adsorbed spores

The range of immune responses obtained in mice immunised with spores pre-adsorbed with GST-Cpa$_{247–370}$, TTFC or PA was examined in more detail. Serum from mice dosed with pre-adsorbed spores in each case seroconverted with high levels of antigen-specific IgG at levels significantly ($P < 0.05$) greater than control groups including those dosed with protein alone (Fig. 1A–D). Antigen-specific secretory IgA (sIgA) was also produced in the lungs, saliva and faeces at levels significantly ($P < 0.05$) greater than control groups demonstrating the induction of mucosal responses as well as systemic (i.e., IgG) (Figs. 6 and 7A–C).

Analysis of isotypes and specifically, an increase in the IgG1:IgG2a ratio was found indicating a T_h2 bias for GST-Cpa$_{247–370}$-specific humoral responses (Supplementary Fig. 2A and B). For PA-adsorbed spores a decrease in PA-specific IgG1:IgG2a ratios was observed (Supplementary Fig. 2C) suggesting a potential T_h1 bias. For TTFC (Supplementary Fig. 2D), levels of both IgG2a and IgG2b were examined only in terminal bleeds but appeared approximately equivalent. IFN-γ was induced in stimulated splenocytes at levels significantly greater than control groups ($P < 0.05$) taken from mice immunised with TTFC or PA-adsorbed spores in agreement with previous studies on TTFC-specific cellular responses [31] (Supplementary Fig. 3) (n.b., IFN-γ was not tested in mice immunised with spores adsorbed with GST-Cpa$_{247–370}$). Taken together, spores pre-adsorbed with antigen could generate a broad range of responses, humoral (mucosal and systemic) and cellular, a characteristic desirable for effective vaccination.

4. Discussion

Bacterial spores have been shown to have utility as recombinant vaccine vehicles where an antigen is expressed on the spore surface or within the germinating spore. As such, spores offer the added advantage of their unique heat-stability and lend themselves to specific applications such as traveller's vaccines or for use in developing countries. As with all recombinant bacterial vaccines there also exist additional concerns over the use of genetically modified microorganisms. This paper reports a specific and novel application of bacterial spores where an antigen can be immobilised on the spore surface and when delivered via a mucosal route generate a broad range of immune responses. More than just stimulating immune responses this approach has been shown to confer protection using two challenge models and a third by inference from the levels of neutralising antibodies. As such spores have the ability to act as mucosal adjuvants without the need for genetic modification.

The single most important concern in the development of adjuvants is safety. *Bacillus* spores, including *B. subtilis*, are being widely used as dietary supplements (i.e., probiotics) with a number of species registered for human use, including *B. subtilis*, *B. cereus*, *B. clausii* [3] and most recently, *B. coagulans*, that has received

GRAS status from the US Food and Drug Administration. Together with the fact that killed spores can be used as effectively as live spores for vaccination strongly supports their consideration as safe, mucosal, adjuvants. Our studies shown here have primarily used the nasal route of administration yet we have also shown that oral delivery can be considered. The size of spores (length of 0.8–1.5 nm depending on species) most closely aligns them with particulate adjuvants where an exogenous antigen displayed on a surface structure closely mimics a potential pathogen in both size and appearance [9,32]. Such particulate adjuvants are easily taken up by antigen presenting cells (APCs) and can induce potent immune responses [9]. Studies have shown that exogenous antigens displayed in this way are presented 1000–10,000 times more efficiently by MHC (major histocompatibility complex) molecules through the class I and class II pathways compared to the soluble antigen alone [33,34]. The broad range of humoral, local and cellular responses observed with immunisation with adsorbed spores are highly desirable in vaccines [32]. The high titres of IgG2a and IFN-γ production are indicative of a T_h1-biased immune responses and the recognition of conformational epitopes [35]. Studies on copolymer adjuvants have lead to the proposal that it is essential to maintain the native conformation of the immunogen to generate protective immunity [11]. Other than the levels of protection achieved in this work we have evidence that a protein when bound to the spore can maintain its native conformation. This arises from studies where we have bound alkaline phosphatase to the spore (at pH 4) and shown that full enzyme activity is retained (unpublished data).

Orally administered spores are taken up by M cells and enter the Peyer's patches where they would be expected to be taken up by APCs [4,36]. We assume that adsorbed spores would also enable delivery of antigens using the same route. It is important to note that recombinant spores also promote the same spectrum of immune responses as adsorbed spores except that with the latter greater amounts of antigen can be loaded per spore. For example, with TTFC, a single intra-nasal dose of recombinant spores will deliver 0.2 μg of immunogen compared to 1 μg using spore adsorption. In addition, adsorbed spores enable antigens to be displayed that ordinarily cannot be expressed on the spore surface, for example, we have succeeded in adsorbing the CSP antigen from *Plasmodium berghei* on the spore which otherwise cannot be expressed (unpublished data).

An important aspect of protein adsorption to spores is that it is enhanced at low pH. As the pH falls below the isolectric point the protein, now carrying a net positive charge would bind to the still negatively charged spore by electrostatic interactions. At pH values above the isoelectric point both protein and spore would be negatively charged thus explaining why binding was minimal or non-existent. One exception, of course, was binding of GST-$Cpa_{247–370}$, which will be discussed later. Adsorption was noticeably enhanced in the presence of salt (at concentrations greater than 1 M) and at high ionic strengths adsorption actually increased. This phenomena has been documented for the adsorption of poliovirus at low pH to membrane filters where both electrostatic and hydrophobic interactions play a role in virus binding [27]. In the virus study salt (NaCl or $MgCl_2$) was shown to enhance hydrophobic interactions and at high ionic strengths (>0.3 M) NaCl could only disrupt electrostatic interactions if hydrophobic interactions were also disrupted. In our study we also showed that treating adsorbed spores with a combination of salt and detergent was most effective at removing a bound protein suggesting a combination of electrostatic and hydrophobic interactions are responsible for binding.

Following binding little dissociation was observed even after resuspension of adsorbed spores in a buffer of higher pH. This stability is important and clearly contributes to the adjuvant effect where spores would encounter the mucus of the nasopharanyx that has a neutral pH. By contrast, oral delivery should further enhance adsorption of antigen to spore but is unable to protect it from the enzymes present in gastric juices. Although we have shown that a protein adsorbed to spores can survive transit through the gastric barrier it seems that the oral route of delivery has limited value.

In data not shown we have also demonstrated that a number of other immunogens can bind to the spore in a pH-dependant manner, including, *Clostridium difficile* toxin A, listeriolysin from *Listeria monocytogenes* and the CSP protein of *Plasmodium berghei*. The one exception was GST-$Cpa_{247–370}$ that although exhibiting strongest binding at low pH, it also bound at a pH greater than its isoelectric point (5.7). It is possible that the GST or Cpa moieties of the chimeric protein fail to adsorb to the spore in their native form and this may explain the resulting Th2-biased immune response.

The surface coat of *B. subtilis* spores is well characterised and consists of about forty different protein species [37]. Spores of most species of *Bacillus* species also carry a well-developed exosporium, a loose fitting glycosylated envelope that encases the spore coat in a sac-like structure [37]. Evidence now emerging has shown that *B. subtilis* may carry a primitive exosporium [38] which may be more apparent in natural isolates of *B. subtilis* and has been observed in spores of HU58 here [39]. What contributes to the net negative charge at low pH is unclear since at this pH carboxylic groups will be fully protonated.

The binding phenomena of spores at low pH is equally relevant at high pH where the spore would become more negatively charged and able to bind positively charged molecules. This application is already being developed for heavy metal adsorption and recovery [40–42]. Other applications apparent from this study are in the adsorption of small peptides, biotherapeutic molecules and enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.10.127.

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