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Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery

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Abstract

We have evaluated the potential of endospores of the Gram-positive bacterium *Bacillus subtilis* as an oral vaccine delivery system. The key features of the *B. subtilis* spore as a vaccine are, non-pathogenicity, advanced cloning tools, extreme robustness, long-term storage properties and its current use as a probiotic for both humans and animals. We have shown previously that the spore germinates in the small intestine of the mouse and have exploited this attribute for heterologous antigen delivery in this work. The first part of this study was to evaluate the fate of spores in vivo as well as under simulated gut conditions. This showed that spores were extremely robust with most being excreted in the faeces. Using a recombinant gene expressing high levels of β-galactosidase specifically in the germinated spore (vegetative cell) we showed that spores administered orally could elicit β-galactosidase-specific local and systemic immune responses. This demonstrated proof of principle that the germinating spore might be effective in the safe delivery of antigens across the stomach barrier. Interestingly, analysis of IgG subclasses suggested a potential bias towards a Th1 response and the involvement of cellular immunity.
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Keywords: *Bacillus subtilis*; Spores; Oral vaccine

1. Introduction

The Gram-positive soil bacterium *Bacillus subtilis* has been extensively studied as a model prokaryotic system with which to understand gene regulation and the transcriptional control of unicellular differentiation [1,2]. This organism is regarded as a non-pathogen and the spore form is currently being used as a probiotic for both human and animal consumption [3]. The use of spores as probiotics prompted some initial studies to examine the fate of spores in the gastrointestinal tract (GIT). In this work, we have shown that spores can germinate in the GIT. This assumption was based on two experimental findings. First, analysis of spore counts in the faeces of mice dosed orally with spores showed that, on occasion, more spores were recovered in the faeces than administered [4]. This, in turn, suggested that spores must have germinated and undergone limited rounds of growth and replication. Second, analysis of expression of two chimeric genes, *ftsH-lacZ* and *rrnO-lacZ*, in the small intestine of mice dosed orally with a pure suspension of spores carrying either of these chimeras [5]. In both cases, each hybrid gene carried a promoter recognised only by RNA polymerase produced during the vegetative phase of cell growth. Analysis of vegetative mRNA using reverse-transcriptase PCR showed that these genes were expressed in the jejunum and ileum and that a proportion of the administered spore dose must have germinated in the small intestine although it was not possible to distinguish between germination in the lumen of the GIT or in the associated tissues.

The spore offers unique resistance properties and can survive extremes of temperature, dessication and exposure to solvents and other noxious chemicals [6]. These unique attributes would make the spore an attractive vehicle for delivery of heterologous antigens or, indeed, any bioactive molecule, to extreme environments such as the gastrointestinal tract.

In this work we have asked whether *B. subtilis* spores have the potential as oral vaccine vehicles using their ability to germinate in the lower GIT as a mechanism for heterologous antigen delivery. Using β-galactosidase as a model antigen we show that spores germinating in the small intestine could provide a simple, yet sophisticated, method for antigen delivery and ultimately for development as mucosal vaccines.

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2. Materials and methods

2.1. Strains

SC2362 has been described elsewhere [5] and carries the *rrnO-lacZ* gene as well as the *cat* gene encoding resistance to chloramphenicol (5 μg/ml). *rrnO* is a vegetatively expressed gene encoding a rRNA. In this strain, the 5′-region of *rrnO* carrying the promoter had been fused to the *E. coli lacZ* gene. PY79 is the prototrophic and isogenic ancestor of SC2362 and is Spo^+ [7]. DL169 (*rrnO-lacZ gerD-cwlBΔ::neo*) was created by transforming competent cells of strain TB1 (*gerD-cwlBΔ::neo*) with chromosomal DNA from SC2362 followed by selection for chloramphenicol resistance carried by the *rrnO-lacZ* cassette. TB1 has the *gerD-cwlB* region of the chromosome replaced with a neomycin-resistance gene and spores of this strain were found to have their rate of germination (as determined by the fall in $OD_{600\,nm}$ in response to addition of germinant; [8]) reduced to 0.0015% when compared to that of an isogenic wild-type strain PY79 (Ricca, personal communication).

2.2. Preparation of spores and vegetative cells

Sporulation was induced in DSM (Difco-sporulation media) media using the exhaustion method as described elsewhere [8]. Sporulating cultures were harvested 22 h after the initiation of sporulation. Purified suspensions of spores were made as described by Nicholson and Setlow [8] using lysozyme treatment to break any residual sporangial cells followed by successive washes in 1 M NaCl, 1 M KCl and then water (two times). PMSF (10 mM) was included in washes to inhibit proteolysis. After the final suspension in water spores were treated at 68 °C for 1 h to kill any residual cells. Next, the spore suspension was titred immediately for cfu/ml before freezing aliquots at −20 °C. Vegetative *B. subtilis* cells were prepared by growth in LB containing 5% D-glucose and 0.2% L-glutamine until an $OD_{600\,nm}$ corresponding to about 10^9 cfu/ml and used immediately. Growth under these conditions prevents inadvertent sporulation [9].

2.3. Analysis of viable bacteria in faecal and intestinal tissues

Faecal counts were made by housing mice individually in cages with gridded floors to prevent coprophagia. Total faeces was collected at appropriate times and homogenised in PBS before plating serial dilutions on DSM agar plates containing chloramphenicol (5 μg/ml) and Xgal (DSMCX; Xgal is 5-bromo-4-chloro-3-indolyl-β-D-galactoside) to select for SC2362 cells. Intestinal tissues were recovered from sacrificed mice and homogenised in PBS using glass beads (0.5 mm; 4 × 30 s bursts, 4 °C) before plating serial dilutions on DSMCX.

2.4. Simulated GIT conditions

Bacteria were grown to a cell density corresponding to approximately 10^9 cells/ml in LB broth, harvested and suspended in simulated gastric juice (1 mg/ml pepsin (porcine stomach mucosa, Sigma), pH 2.0) or small intestine fluid (0.2% bile salts (50% sodium cholate: 50% sodium deoxycholate; Sigma), pancreatin (1 μg/ml), pH 7.4). The suspensions were incubated at 37 °C, samples removed, serially diluted and plated for cfu/ml on LB agar plates.

2.5. Indirect ELISA for detection of β-galactosidase-specific serum antibodies

Plates were coated with 50 μl per well of purified β-galactosidase (Sigma, 2 μg/ml in carbonate/bicarbonate buffer) and left at room temperature overnight. After blocking with 2% BSA in PBS for 1 h at 37 °C serum samples were applied using a two-fold dilution series starting with a 1/40 dilution in ELISA diluent buffer (0.1 M Tris–HCl, pH 7.4; 3% (w/v) NaCl; 0.5% (w/v) BSA; 10% (v/v) sheep serum (Sigma); 0.1% (v/v) Triton-X-100; 0.05% (v/v) Tween-20). Every plate carried replicate wells of a negative control (a 1/40 diluted pre-immune serum), and a positive control (mouse anti-β-galactosidase (Sigma)). Plates were incubated for 2 h at 37 °C before addition of anti-mouse HRP conjugates (Sigma). Plates were incubated for a further 1 h at 37 °C then developed using the substrate 3,3′,5,5′-tetramethyl-benzidine (TMB; Sigma). Reactions were stopped using 2 M H_2SO_4. Dilution curves were drawn for each sample and endpoint titres calculated as the dilution producing the same optical density as the 1/40 dilution of a pooled pre-immune serum. Statistical comparisons between groups were made by the Mann–Whitney *U*-test. A *P*-value of >0.05 was considered non-significant. To measure faecal IgA, a similar ELISA protocol was followed as described previously [10]. Samples were applied using a two-fold serial dilution starting with undiluted faecal extract in PBS/2% BSA/0.05% Tween-20. End-point titer was calculated as the dilution producing the same optical density as the undiluted pre-immune faecal extract. An end-point titer of 6.0 or greater was considered "positive".

2.6. Extraction of spore coat proteins and vegetative cell lysates

Spore coat proteins were extracted from suspensions of spores of strain PY79 at high density (1×10^{10} spores/ml) using an SDS–DTT extraction buffer as described in detail elsewhere [8]. For vegetative cell lysates, strain PY79 was grown to an $OD_{600\,nm}$ of 1.5 in LB medium and the cell suspension washed and then lysed by sonication followed by high speed centrifugation. Extracted proteins were assessed for integrity by SDS–PAGE and for concentration using the Bio-Rad DC Protein Assay kit.

2.7. Immunisations

Groups of eight mice (female, BALB/c, 8 weeks) were dosed orally with suspensions (0.2 ml) of either spores or vegetative cells of either strain PY79, SC2362 or DL169. Mice were lightly anaesthetised with halothane. A naïve, non-immunised control group was included. Oral immunisations were administered by intra-gastric gavage on days 0, 1, 2, 20, 21, 22, 41, 42 and 43. Serum samples were collected on days −1, 18, 40 and 60, and fresh fecal pellets were collected on days −1, 18, 40 and 58. Faecal samples (0.1 g) were incubated overnight at 4 °C in 1 ml PBS/1% BSA/1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma), then vortexed to disrupt all solid materials, and centrifuged at 13,000 rpm for 10 min. Sera and faecal extracts were stored at −20 °C until required.

2.8. Immunofluorescence microscopy

B. subtilis strains (PY79 and SC2362) were grown to mid-log in LB medium. Samples were fixed in situ as described elsewhere [11]. The glass coverslips containing fixed samples were probed with mouse anti-β-galactosidase and anti-mouse IgG–TRITC conjugate antibodies (Sigma) following the protocol of [12]. The coverslips were then mounted onto a microscope slide and viewed under a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system. Images were taken using LaserSharp software and processed with the Confocal Assistant Programme. Laser power was 30% for Green HeNe, scanning speed was 50 lps. Image size was 10 μm × 10 μm.

3. Results

3.1. Survival of B. subtilis in the gastrointestinal tract

As a first step in developing spores for heterologous antigen delivery via the oral route we assessed the survival of *B. subtilis* vegetative cells and spores in the gastrointestinal tract of a murine model. To assess the robustness of the vegetative cell we inoculated two groups of inbred mice (BALB/c) each with a single dose of 2.4×10^{10} vegetative cells of strain SC2362 (*rrnO-lacZ*). One group of six mice was assessed for the number of viable counts of SC2362 that were present in the faeces collected from individually housed mice for the first 24 h after dosing (Fig. 1A). In this study, the *rrnO-lacZ* marker enabled simplified identification and screening of viable colonies using the Lac^+ phenotype as well chloramphenicol resistance (encoded by the *cat* gene and carried by the *rrnO-lacZ* construct). Maximal counts of SC2362, corresponding to 0.00016% of the original dose, were found 6 h after dosing and these declined rapidly thereafter to insignificant levels by 24 h. The mean cumulative counts of SC2362 recovered in the faeces in the

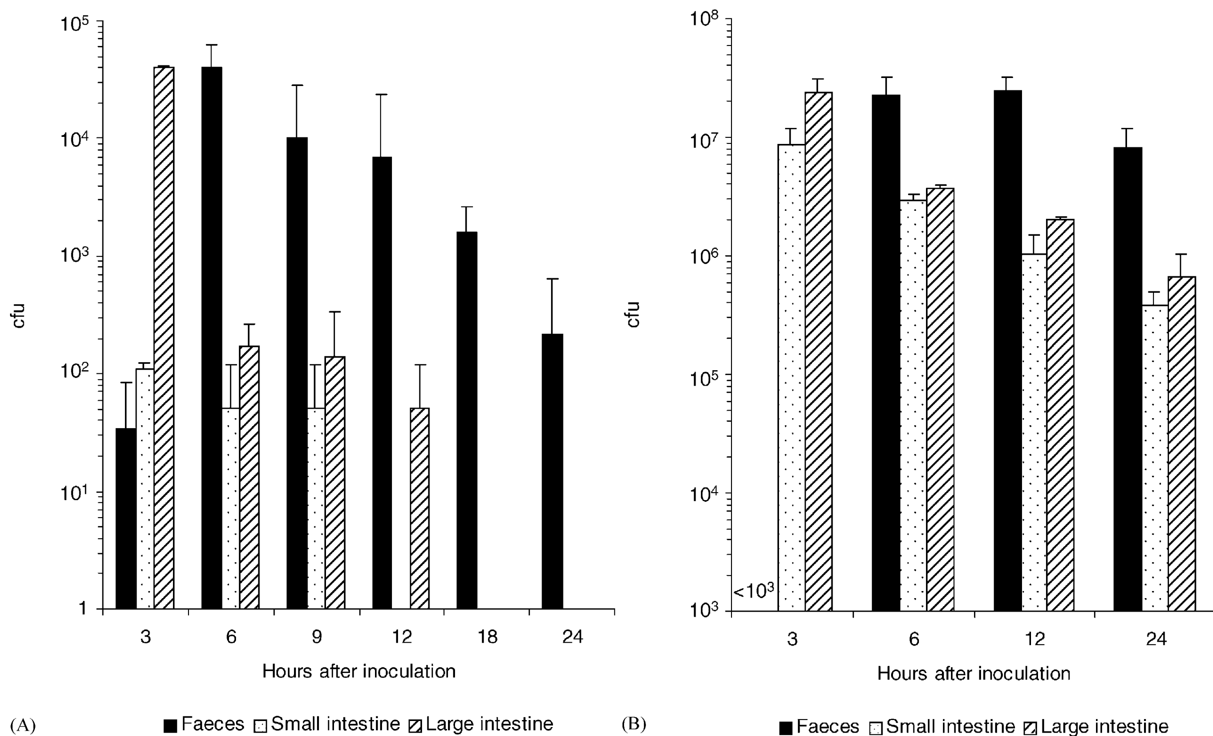

Fig. 1. Survival of vegetative cells vs. spores in GIT of a mouse model. Groups of inbred BALB/c mice were orally dosed with vegetative cells or spores of *B. subtilis* strain SC2362. Faecal and intestinal samples were assessed for total viable counts at indicated time points. (A) Oral dose of 2.4×10^{10} vegetative cells; (B) oral dose of 2.1×10^{8} spores. Data were presented as arithmetic means and error bars were standard deviations.

first 24 h corresponded to 0.00025% of the inoculating dose. With the second group of mice (12 animals) two animals were sacrificed at 3, 6, 9, 12, 18 and 24 h and the small and large intestines were removed, homogenised and plated for counting of SC2362 viable units. As shown in Fig. 1A, very low numbers of SC2362 were found in the small intestine with maximal counts being found at 3 h (approximately 100). Higher counts (0.00016% of inoculating dose) were found in the large intestine at 3 h but these counts declined thereafter.

To examine spore survival we performed a similar experiment to that described above but dosing orally with 2.1×10^8 spores of strain SC2362 per mouse (Fig. 1B). Our assay technique differed from a previous study [4] in that the faeces was not heat-treated before plating and thus counts would include both spores as well as germinated spores (vegetative cells). Faecal counts from a group of six mice showed viable SC2362 present in the faeces at 6 h in significant numbers with maximum levels at 12 h (∼12% of inoculating dose). By 24 h, there were still considerable numbers of SC2362 counts (∼4%) present in the faeces. Counts in the small and large intestines showed similar kinetics as with dosing with vegetative bacteria (with maximal counts at 3 h) but with significantly higher levels of viable units. A group of five mice was also used as a naïve control of which one mouse was examined for faecal counts and the other four examined at appropriate time points for analysis of small and large intestinal counts. In each case, no counts were recovered validating our assay technique.

3.2. Survival of B. subtilis in simulated GIT environments

We next asked what effect conditions within the GIT would have on the survival of both vegetative *B. subtilis* and intact spores using an in vitro assay. Based upon previous studies simulating conditions within the GIT [13–17] we recreated two environments, stomach and small intestine. Simulated conditions found in the stomach consisted of pepsin (1 mg/ml) at pH 2.0 in LB medium, and for the small intestine, 0.2% bile salts and pancreatin (1 mg/ml) at pH 7.4 in LB. For assessing survival of spores though, LB was replaced with PBS since the nutrient rich LB medium might promote spore germination. Suspensions of vegetative *B. subtilis* cells or spores of strain SC2362 at approximately 10^8–10^9 cfu/ml were incubated in simulated stomach or small intestine conditions at 37 °C and survival determined by plating out and determination of cfu/ml. We also included two enteric bacterial species as controls, *E. coli* (strain BL21) and *Citrobacter rodentium* (ATCC 51459) the latter being a mouse pathogen that infects the small intestine [18].

As shown in Fig. 2, simulated gastric conditions resulted in a significant reduction in viability of vegetative cells of *B. subtilis* (Fig. 2A), *E. coli* (Fig. 2B) and *C. rodentium*

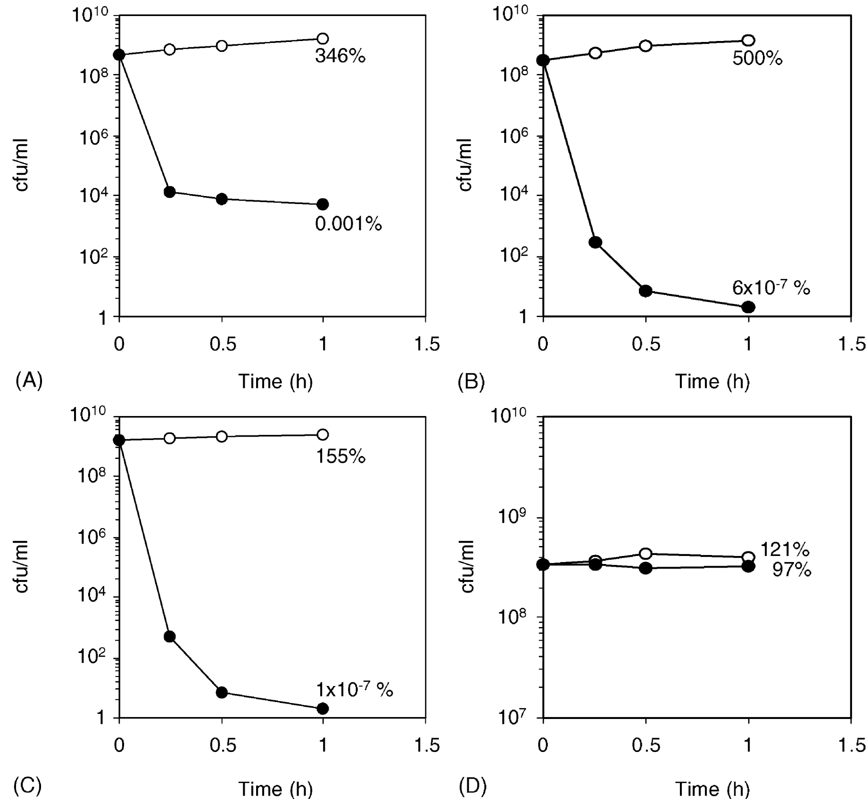

Fig. 2. Survival of vegetative cells and spores in simulated gastric condition. Vegetative cells of *B. subtilis*, *E. coli*, *C. rodentium*, and spores of *B. subtilis* ((A–D), respectively) were treated (●) in simulated gastric conditions, and viability was assessed at indicated time points in comparison with untreated (○) samples. Percentages were counts compared to original inocula. Data were presented as arithmetic means of duplicate independent experiments.

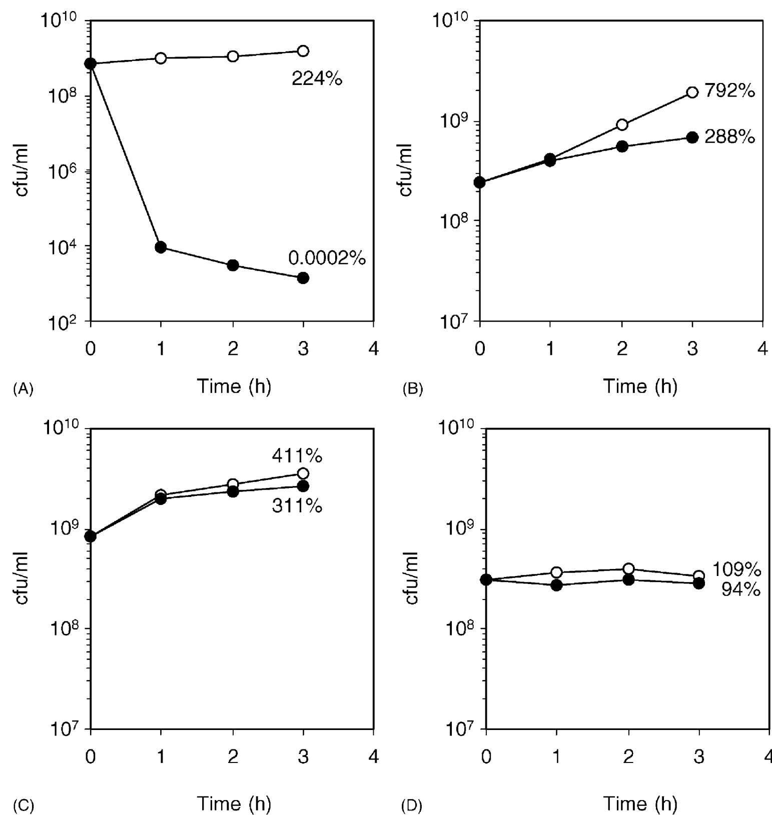

Fig. 3. Survival of vegetative cells and spores in simulated intestinal condition. Vegetative cells of *B. subtilis*, *E. coli*, *C. rodentium*, and spores of *B. subtilis* ((A–D), respectively) were treated (●) in simulated intestinal condition, and viability was assessed at indicated time points in comparison with untreated (○) samples. Percentages were counts compared to original inocula. Data were presented as arithmetic means of duplicate independent experiments.

(Fig. 2C) with almost complete loss of viability within 1 h. Spores were essentially unaffected though (Fig. 2D). Bile salts found in the small intestine were found to have a significant effect on the viability of vegetative *B. subtilis* with only 0.0002% of the original inoculum surviving after the first hour (Fig. 3A). *E. coli* and *C. rodentium* however, were unaffected and could grow under these conditions with a moderate increase in cell numbers (Fig. 3B and C). The effects on *B. subtilis* though, are primarily due to bile salts since in the absence of pancreatin cell viability was still substantially reduced to almost the same levels (data not shown). Finally, bile salts appeared to have no effect on intact spores (Fig. 3D).

3.3. Spore germination in simulated intestinal conditions

Upon entry into the duodenum spores have been shown to germinate [4,5]. Since this region is rich in bile salts and our work has shown an effect of bile salts on cell viability we wondered what effect bile would have on spore germination. Using established procedures [8] we assessed germination in the presence or absence of 0.2% bile salts. A suspension of pure spores (wild-type strain PY79) was incubated at 37 °C in the presence of specific germinants referred to as alanine–glucose–KCl (AGK) with L-alanine serving as a universal germinant for *B. subtilis* [19]. L-Alanine was added at 10 mM (final concentration) to trigger spore germination and $OD_{600\,nm}$ readings taken (Fig. 4). As spores germinate the OD declines as phase-bright spores lose their refractility and outgrow [20,21]. Our results (repeated two times) showed that in the presence of AGK spore germination was extremely rapid with a drop to 32.4% in $OD_{600\,nm}$ (relative to initial $OD_{600\,nm}$) in the first 90 min. In the presence of 0.2% bile salts though, spore germination was inhibited but not abolished with a drop to 42.8% in $OD_{600\,nm}$ over 90 min. Although we have not tested germination in the presence of L-asparagine (another known spore germinant, [19]) the inhibitory action of bile salts on L-alanine-mediated spore germination has been observed previously [22] and is consistent with our more detailed findings here.

3.4. Spores as an antigen delivery vehicle

Our studies here demonstrate that spores are well equipped to survive transit across the stomach barrier. To address whether the spore could be used for heterologous antigen delivery we made use of the *rrnO-lacZ* gene carried in SC2362. *rrnO-lacZ* is itself a chimeric gene containing the strong, σ^A-recognised *rrnO* promoter fused to the *lacZ* gene of *E. coli* [5]. As a control, we constructed a

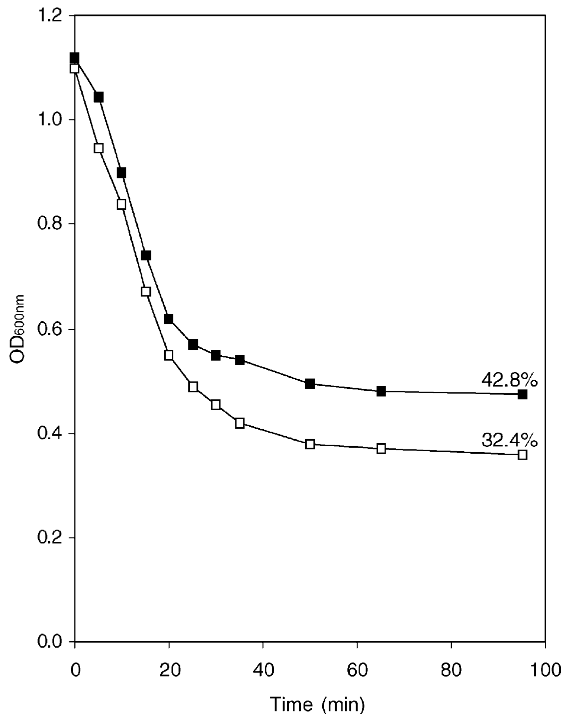

Fig. 4. Spore germination in simulated intestinal condition. Spore suspensions of *B. subtilis* strain PY79 were examined for germination in AGK solution with (■) or without (□) the presence of bile salts. $OD_{600\,nm}$ readings were taken at indicated time points following the addition of L-alanine to trigger germination. Percentages were of OD readings compared to original suspensions. Data are presented as arithmetic means of duplicate independent experiments.

germination mutant, DL169, which carried *rrnO-lacZ* together with a deletion (*gerD-cwlBΔ::neo*) in the *gerD-cwlB* region of the chromosome which is important for spore germination. Spores carrying the *gerD-cwlB* deletion are severely impaired (reduced to 0.0015% of wild-type spores) in their ability to germinate (Ricca, personal communication). We verified that *lacZ* was expressed in vegetative cells of SC2362 by immunofluorescence as shown in Fig. 5A using a polyclonal sera against β-galactosidase. No detectable expression was found in the isogenic wild-type strain PY79. SDS–PAGE analysis of fractionated whole cell extracts of SC2362 and DL169 cells (Fig. 5B) revealed a predominant band at 117 kDa corresponding to the size of β-galactosidase. Western blotting with a polyclonal anti-β-galactosidase antibody confirmed this and showed a number of high molecular weight breakdown products but otherwise no obvious degradation.

A quantitative determination of the amount of β-galactosidase expressed in SC2362 cells expressing *rrnO-lacZ* was obtained by dot blot experiments using serial dilutions of purified β-galactosidase (Sigma) and of whole cell extracts of *B. subtilis* strains PY79, SC2362 and DL169 (Fig. 5C). Proteins were reacted with an anti-β-galactosidase polyclonal antibody, then with alkaline phosphatase-conjugated secondary antibodies and colour developed by the BCIP/NBT or ECL system (Bio-Rad). A densitometric analysis indicated that no β-galactosidase was detectable in PY79 cells. In SC2362 and DL169 cell extracts, the amount of β-galactosidase equated to 3.14% (31.4 ng/μg) of total

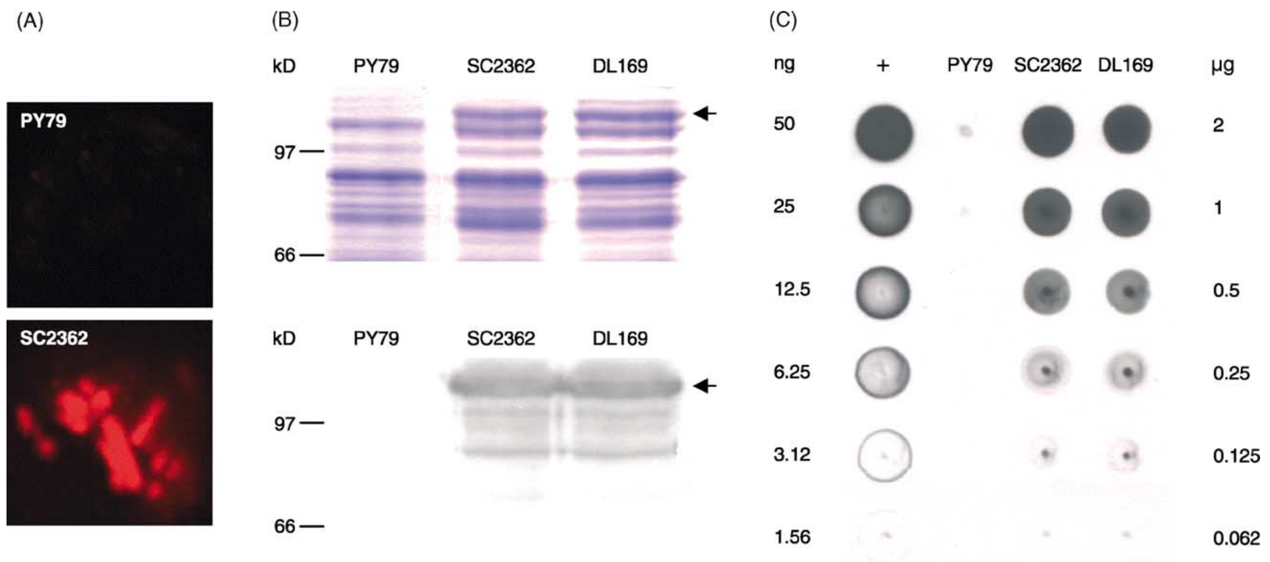

Fig. 5. Expression and quantification of expressed β-galactosidase. (A) Samples of PY79 and SC2362 (*rrnO-lacZ*) grown in LB were labelled with mouse anti-β-galactosidase antibody followed by anti-mouse IgG–TRITC conjugate (red fluorescein). (B) Coomassie stained 10% SDS–PAGE (upper panel) and β-galactosidase-specific Western blot (lower panel) profiles of fractionated cell extracts from PY79 (spo^+), SC2362 (*rrnO-lacZ*) and DL169 (*gerD-cwlBDΔ::neo rrnO-lacZ*). Arrows indicate β-galactosidase at the predicted molecular weight of 117 kDa. (C) Dot blot experiments performed with the indicated amounts of cell extracts (in μg) from strains PY79 (spo^+), SC2362 (*rrnO-lacZ*) and DL169 (*gerD-cwlBDΔ::neo rrnO-lacZ*). Purified β-galactosidase dilutions (in ng) are spotted on the left (lane +). Anti-β-galactosidase primary antibodies and secondary anti-rabbit peroxidase-conjugated antibodies were used. Reactions were visualised by ECL as described in Section 2.

extracted protein for SC2362 and 2.4% (24 ng/μg) of total extracted protein for DL169. The high levels of β-galactosidase produced in these strains were confirmed by the SDS–PAGE analysis (Fig. 5B) and demonstrate the efficacy of the *rrnO* promoter for heterologous gene expression. Spores of SC2362 and DL169 carried no detectable β-galactosidase as determined by Western blotting or by immunofluorescence showing that no residual β-galactosidase could accumulate within the spore (data not shown).

3.5. Serum anti-β-galactosidase responses following oral delivery of spores carrying rrnO-lacZ

Groups of seven inbred mice were dosed orally with spores or vegetative cells of SC2362, DL169 or PY79. We used a dosing regime previously optimised for oral immunisations [10] and each immunising dose contained either 2×10^{10} spores or 3×10^{10} vegetative cells. From our densitometric analysis (see Section 3.4) we could define one dose of SC2362 or DL169 vegetative cells as containing approximately 0.43 mg of β-galactosidase. Serum samples were analysed by ELISA for anti-β-galactosidase IgG (Fig. 6) and as a control we also included a group of seven non-immunised mice for sampling. As shown in Fig. 6, oral immunisation of mice with SC2362 (*rrnO-lacZ*) spores gave end-point titres significantly above ($P < 0.05$) those of mice dosed with non-recombinant spores (PY79) or the control naïve group from day 40 onwards. DL169 spores, though, failed to produce seroconversion in immunised mice and anti-β-galactosidase titres did not significantly ($P > 0.05$) differ from those of mice dosed with non-recombinant spores (PY79) or the control naïve group. This then shows clearly that a proportion of SC2362 spores must have germinated following oral delivery leading to subsequent expression of *rrnO-lacZ*. Failure to generate these responses following delivery of DL169 spores proves that spore germination is essential for generating these humoral responses. At this stage, we are not concerned with the levels of antibody responses but rather proof that spore germination can be used for antigen delivery.

Immunisations using vegetative cells of each strain were incorporated as controls and we were somewhat surprised to detect anti-β-galactosidase IgG responses in mice dosed with SC2362 or DL169 cells. The levels of responses were similar to those obtained from dosing with SC2362 spores (Fig. 6) and the similarity in responses may imply a threshold level has been reached when using this dosing regime and with β-galactosidase as the immunogen.

Sera from mice immunised with SC2362 spores (Fig. 7A), SC2362 vegetative cells (Fig. 7B) and DL169 vegetative cells (Fig. 7C) was also examined for the presence of β-galactosidase-specific IgG1, IgG2a and IgG2b subclasses. Immunisation with vegetative cells of either SC2362 or DL169 showed IgG2a to be the first detectable subclass at day 20 followed by a gradual increase in IgG1. Dosing

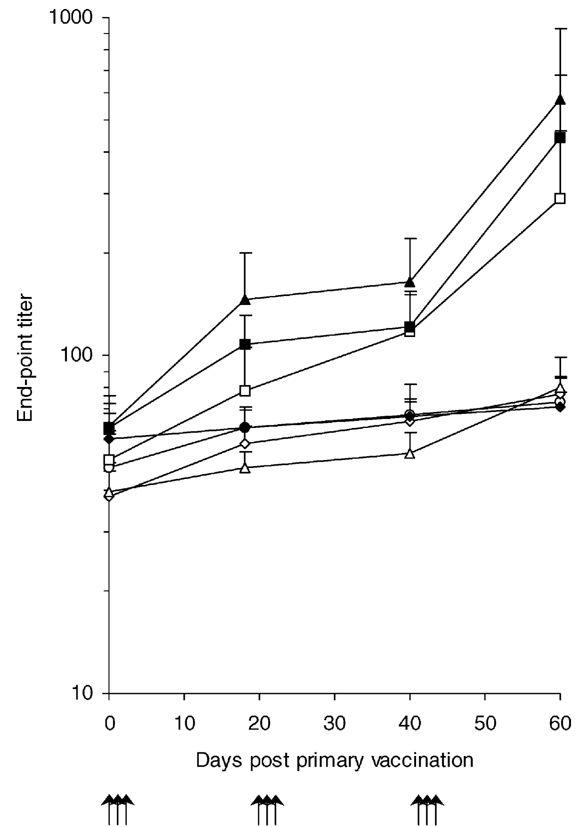

Fig. 6. Systemic responses after oral delivery of spores carrying *rrnO-lacZ* gene. Groups of inbred BALB/c mice were orally dosed (↑) with 2×10^{10} spores per dose or 3×10^{10} vegetative cells per dose of *B. subtilis*. Individual serum samples were tested by ELISA for anti-β-galactosidase-specific IgG. Sera from a naïve, non-immunised, control group (○) were also included as well as mice dosed with PY79 spores (◇), PY79 vegetative cells (◆), SC2362 spores (□), SC2362 vegetative cells (■), DL169 spores (△), and DL169 vegetative cells (▲). Data were presented as arithmetic means and error bars were standard deviations.

with SC2362 spores showed an early increase in both IgG1 and IgG2a. In all three cases the levels of IgG2b increased more slowly.

3.6. Mucosal anti-β-galactosidase responses following oral delivery of spores carrying rrnO-lacZ

IgA recovered from faeces (see Section 2) was used to determine mucosal immune responses. Out of eight mice, only one in the group receiving SC2362 spores gave a positive response of (an sIgA titre of 16.8 on day 58). The level of anti-β-galactosidase-specific faecal IgA in the group immunised with vegetative cells of the same strain was higher with 3, 1 and four out of eight mice showing positive responses on days 18, 40 and 58, respectively (data not shown). Finally, the group immunised with DL169 spores gave no positive responses and with DL169 vegetative cells only one positive response on day 18. No positive titers were found with other groups.

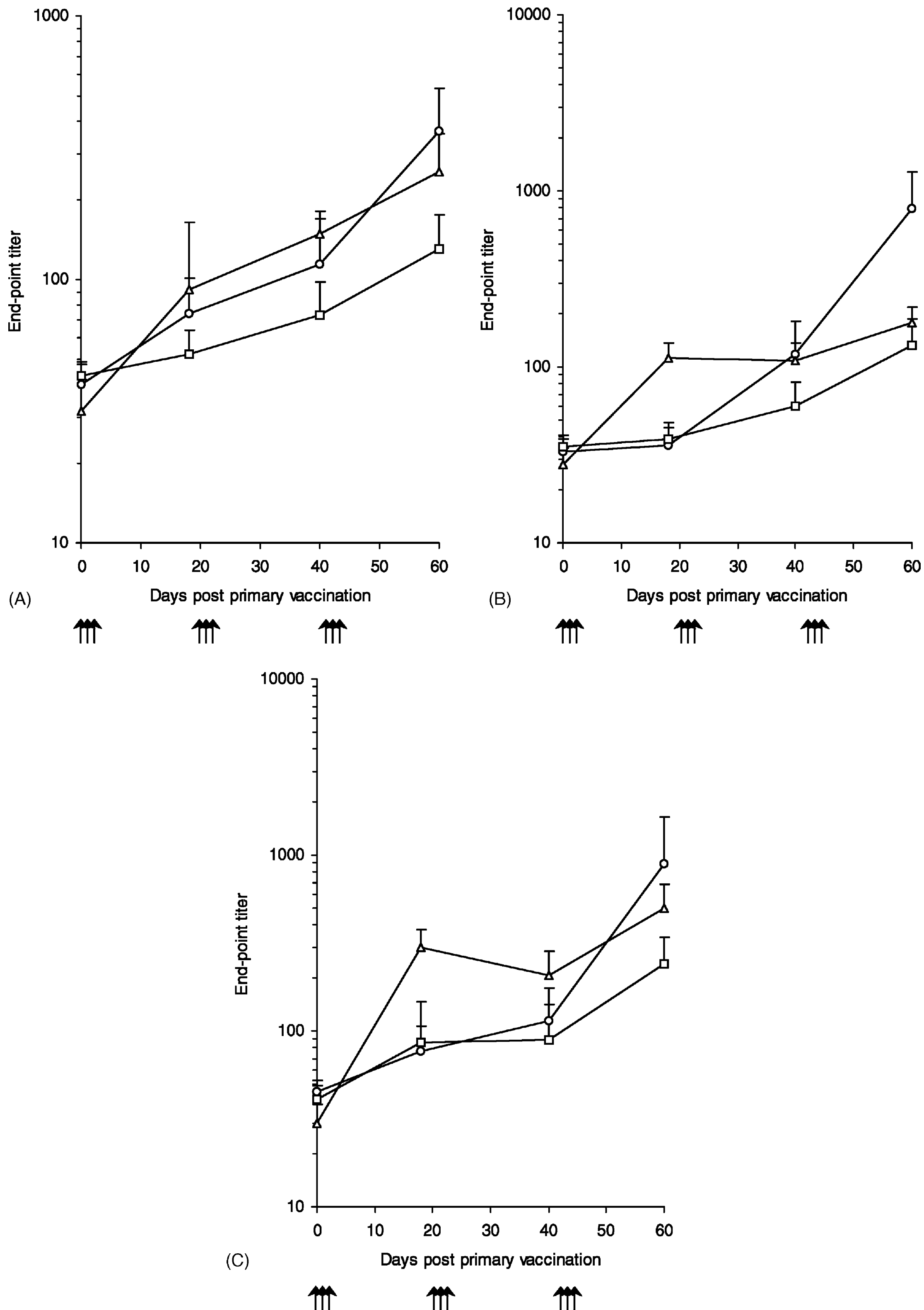

Fig. 7. Analysis of anti-β-galactosidase IgG subclasses. Groups of inbred BALB/c mice were orally dosed (↑) with 2×10^{10} spores per dose of *B. subtilis* strain SC2362 (A), or 3×10^{10} vegetative cells per dose of strain SC2362 (B), or vegetative cells of strain DL169 (C). Individual serum samples were tested by ELISA for anti-β-galactosidase-specific IgG1 (○), IgG2a(△), and IgG2b (□) subclasses. Data were presented as arithmetic means and error bars were standard deviations.

4. Discussion

The aim of this study was to evaluate *B. subtilis* spores as an oral vaccine delivery system. Our rationale was based on several attributes that would make spores a particularly promising vaccine vehicle. First, their current use as a probiotic for human and animal use. Second, they are non-pathogenic micro-organisms normally found in the soil. Third, as robust and dormant life forms they would be suitable for long-term storage in the dessicated (spore) form. Fourth, as a model unicellular differentiating (spore-forming) organism genetic analysis in this organism is second to none and supported by excellent cloning technology. Finally, this organism when administered orally in the spore state can germinate and undergo limited rounds of replication and cell growth in the small intestine before being excreted. Based on the ability of spores to germinate in the GIT we have investigated the germinating spore as the mechanism for heterologous antigen delivery. The logic and novelty of our approach is that the spore might be able to survive transit across the stomach after which it would germinate and then in the vegetative phase express the heterologous antigen.

Before evaluating specific humoral responses we evaluated the survival of spores as well as vegetative cells in the GIT tract. Using an in vivo analysis in mice we have found that spores are essentially unaffected when given orally with most being excreted after 24 h. By contrast, vegetative *B. subtilis* cells have a very low survival in the mouse GIT. As an approximation we estimate less than 0.0005% of vegetative cells survive transit through the GIT. The stomach would likely be the first and most severe barrier to vegetative *B. subtilis* and this is supported by the extremely low levels of viable counts recovered in the small intestine. For those bacteria that do survive they appear to have transited the stomach within the first 3 h after dosing and were present in the faeces by the sixth hour.

We supported these observations by using an in vitro assay where spore or vegetative cell survival was assessed in simulated conditions mimicking the stomach or small intestine. These results showed that for vegetative *B. subtilis* there is a limited chance for long-term survival in the stomach or small intestine. The simulated stomach environment appeared to present a hostile environment not only to *B. subtilis* but also to other enteric bacteria such as *E. coli* and *C. rodentium*. For *B. subtilis*, this is shown from our direct counting experiments of small intestine tissues where clearly some percentage of cells have survived transit across the stomach. Presumably, in vivo, survival is due to the effects of clumping and aggregation, transit time and the composition the stomach. For *B. subtilis*, a second barrier comprised of the effects of bile salts is presented upon exit from the stomach which would ensure almost no survival and is supported by our in vivo experiments described above where we estimate less than 0.0005% of vegetative bacteria can survive transit through the GIT. Spores, as might be expected, can survive such harsh conditions with no deleterious effect.

The effect of bile salts on *B. subtilis* demonstrate the inability of this organism to survive, long-term, in the GIT, in contrast to enteric microbes. The effect of bile salts on spores and vegetative cells was interesting. While bactericidal on vegetative cells their effect on spores was a modest inhibition of germination. Thus, spores exiting the stomach would initially be inhibited from germinating but those that did germinate would be eventually killed. These opposed effects though, would be modulated by the precise composition of the intestinal lumen as well as the distance passed after exit from the stomach. Despite the inhibitory effect of bile we know from previous studies that at least a percentage of spores must be able to germinate in the small intestine and L-alanine (and other germinants such as L-asparagine) would be expected to be present in this nutrient rich region of the intestine [4,5].

We have used β-galactosidase as the model antigen to evaluate our vaccine hypothesis since this protein has been used successfully to evaluate new vaccine delivery systems [23,24]. Our analysis of systemic anti-β-galactosidase IgG responses to orally delivered spores proves that spores can indeed germinate and synthesise sufficient immunogen to generate the observed seroconversion. This would validate our hypothesis and demonstrate the potential of spores as vaccine vehicles. Our previous molecular studies [5] suggest that a proportion of spores can germinate in the small intestine and presumably these enter the GALT at this region. Alternatively, intact spores may transit the mucosa and germinate within the GALT (e.g. in the Peyer's Patches). The small size (1–1.2 μm) of the spore particle make this a distinct possibility since they are small enough to be taken up by M cells. Generation of secretory IgA responses are obviously beneficial for any mucosal vaccine and local responses to β-galactosidase were low in this pilot study although some mice did show responses. Most probably this reflects the relatively low immunogenicity of β-galactosidase but might also reflect the dosing regime. Interestingly, we obtained similar responses when vegetative cells were used for antigen delivery. These were used as controls yet despite the fact that we predicted almost 100% cell death in the stomach sufficient β-galactosidase could be delivered to generate the observed anti-β-galactosidase IgG titres. We can estimate the oral dose of antigen as approximnately 0.43 mg which was given nine times. Presumably, the responses we observe come from intact vegetative cells that have transited the stomach and entered the small intestine which is responsible for generating humoral responses of orally administered antigens. We cannot say from this study whether killed *B. subtilis* cells can generate the observed humoral responses but we would predict that it does not matter whether the cell is alive or dead. We base this on a previous study examining the humoral responses generated against orally delivered tetanus toxin fragment C (TTFC) antigen expressed within *Lactococcus lactis* [10]. In this

work, it was shown that similar anti-TTFC IgG titres could be obtained using live bacteria or formalin killed bacteria. Presumably, then, the bacterium so long as it is not lysed, can simply encapsulate the expressed antigen and allow safe passage through the stomach without cell lysis. This issue is not of importance here though since we propose only to use the spore form as the vaccine delivery system. At first glance, it might not seem obvious why the spore state is advantageous since both forms can induce local and systemic responses. The spore state though, offers the benefits of long-term storage (perhaps in terms of decades) in the dessicated state at ambient temperature.

These studies are the first stages in our development of bacterial spores as vaccine vehicles. Using a model immunogen we have proven principle and we are now developing spores further as specific mucosal vaccines. While our studies here are confined to *B. subtilis*, it seems probable that other spore formers could be used with equal or better effect. While we have only evaluated humoral responses here we have some evidence for cellular immunity. This comes from our analysis of anti-β-galactosidase subclasses where we observed an indication of a predominance of the IgG2a subclass over IgG1 during the early stages of immunisation. Compelling evidence shows that a predominance of this subclass is indicative of a type 1 (Th1) T-cell response leading to CTL recruitment as well as IgG synthesis [10,25–28] The increase in IgG2b during the later stages of immunisation indicates a bias towards a type 2 (Th2) T-cell response. We are investigating the nature of cellular responses but involvement of cellular as well as humoral immunity is a positive indicator for a good vaccine.

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