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Enhanced vaccine antigen delivery by *Salmonella* using antibiotic-free operator–repressor titration-based plasmid stabilisation compared to chromosomal integration

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ABSTRACT

Live attenuated bacteria provide the potential to replace traditional needle-based vaccination with an orally administered vaccine. The heterologous antigen gene is usually transformed as a multi-copy plasmid into the bacterial cell, but plasmids in live bacterial vaccine strains are often unstable, so an alternative approach is to integrate the single-copy antigen gene into the bacterial chromosome. We report a comparison between the chromosomally integrated and the plasmid-borne *Bacillus anthracis* protective antigen gene in live *Salmonella enterica* serovar Typhimurium, using the Operator–Repressor Titration (ORT) system to ensure stable plasmid maintenance. These studies demonstrate that the stabilised plasmid approach of gene expression produced greater amounts of antigenic protein, which in turn resulted in higher antibody responses and levels of protection in mice.

1. Introduction

Live attenuated bacteria such as *Salmonella* spp. are promising vectors for the delivery of heterologous vaccine antigens, offering the advantage of mucosal immunisation via the oral route and relieving the logistical problems associated with needle-based injection of vaccines. Such an advantage makes the use of recombinant live attenuated bacterial vaccines attractive for both general disease control [1], veterinary and specifically for biodefence purposes [2]. While there are currently no heterologous antigen-expressing live attenuated bacterial vaccines licensed for human use, there is already a precedent for the use of live attenuated *Salmonella* in humans: the typhoid vaccine strain *Salmonella enterica* serovar Typhi Ty21a. A number of recombinant *Salmonella* vaccines are in clinical trials [3].

An important problem encountered in the development of *Salmonella* vaccines is the instability of heterologous antigen-encoding plasmids within the bacterial cells. Thus, approaches to solving the instability and segregational loss of the multi-copy antigen-encoding plasmids are being sought, including balanced-lethal [4–6] and post-segregational killing [7] systems. Recently, we have demonstrated the use of an alternative antibiotic-free gene stabilisation technology that offers several advantages over these systems, including the lack of requirement for selectable marker genes on the plasmid. This technology, Operator–Repressor Titration (ORT) was adapted for use in live vaccine vectors and used to stabilise high copy number plasmids in *Salmonella*. In addition, we have successfully applied the ORT technology to vaccination of mice with a low copy number *Salmonella*-based vaccine against bubonic plague [8]. The mechanism of ORT in the work presented utilises an essential chromosomal gene, *dapD*, which catalyses a step in the lysine/diaminopimelate (DAP) pathway. DAP is essential for stabilising peptidoglycan in the cell wall: *dapD* mutants lyse in its absence, and it is not found in standard bacterial growth media or in mammalian tissues. In ORT strains, the native *dapD* promoter is replaced with the lactose repressor and operator/promoter (*lacO*/*P*), such that growth of an untransformed bacterium is dependant on the presence of an inducer such as IPTG. When the bacterium is transformed with a multi-copy plasmid containing a *lac* operator, the repressor binds to the plasmidal *lacO* and therefore *dapD* is derepressed, enabling the growth and selection of transformants on LB agar plates.

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However, an alternative approach for solving the gene stability problem is via the integration of the heterologous antigen-encoding genes into the chromosome of the *Salmonella* vector [9–12]. Therefore to determine the optimum gene stabilisation strategy and further demonstrate the application of ORT technology to live bacterial vector vaccines (ORT-VAC), we describe here a comparison between *Salmonella* strains expressing the *Bacillus anthracis* protective antigen (PA) vaccine from an ORT-stabilised plasmid and a single-copy chromosomally integrated gene.

2. Results

2.1. Construction of Zoosaloral H strains stably expressing PA

The strain of *S. enterica* serovar Typhimurium used in these studies was Zoosaloral H, an attenuated poultry vaccine strain [13]. Two derivative strains were created to express the anthrax protective antigen gene: a chromosomal integrant possessing a single gene copy (Z-pagPA), and an ORT-VAC strain with the PA gene on a multi-copy plasmid (Z-ORT/pSCpagPA). The cassette constructed for exporting PA contained the *PA83ec* gene (codon-optimised for *E. coli*) fused to the region encoding the C-terminus of the haemolysin gene *hlyA*. Accessory genes *hlyBD* enabled export of the PA83ec-HlyA protein from the cell: HlyB is an inner membrane protein that drives export using ATP hydrolysis through HlyD, which forms a pore between the inner and outer membranes by interacting with the native outer-membrane protein TolC, bypassing the periplasm and allowing the export of HlyA [14]. Expression of all three cistrons was regulated by the promoter *pagC*, which is induced in the phagosome. The Zoosaloral H-based ORT strain, Z-ORT, was produced by placing the *dapD* gene under the control of a modified *lac* operator–promoter and introducing a $lacI^q$ repressor gene, as described in Garmory et al. [8]. A *PA83ec-hlyA* expression plasmid, pSCpagPA, was constructed with the *lac* operator sequence (*lacO*) but no selectable marker gene (Fig. 1A). Prior to transformation of Z-ORT, the Lac repressor protein binds to the chromosomal *lacO* and prevents the expression of the essential *dapD* gene, leading to cell lysis on LB agar plates. However, when transformed with pSCpagPA (or any plasmid containing the *lac* operator), the plasmid titrates the repressor and enables *dapD* expression, and therefore plasmid selection and maintenance. The integrant strain Z-pagPA was engineered using the Xer-cise system [15], with the chloramphenicol resistance gene used to select for initial integration flanked by *dif* sites, enabling its subsequent excision by the native Xer site-specific recombinases in the absence of chloramphenicol. This resulted in the insertion of the *PA83ec-hlyA* gene cassette with no antibiotic resistance gene remaining in the chromosome (Fig. 1B). The integration left the flanking genes *fadA* and *SMT3981* intact. Thus Zoosaloral H-based strains free of antibiotic resistance genes were created with identical *PA83ec-hlyA* fusion genes either on a multi-copy plasmid (Z-ORT/pSCpagPA) or integrated into the chromosome (Z-pagPA).

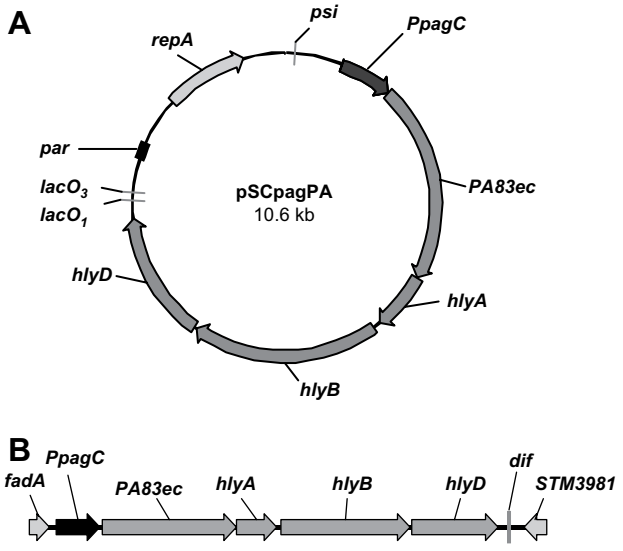

Fig. 1. The PA-expressing ORT plasmid pSCpagPA (A), and PA-expressing cassette integrated into the *fadA-STM3981* chromosomal locus of strain Z-pagPA (B).

Growth studies indicated that Zoosaloral H, Z-pagPA and Z-ORT/pSCpagPA had identical growth profiles, indicating that there is no reduction in viability *in vitro* as a result of the genetic manipulations performed (data not shown). Serial subculture of Z-ORT/pSCpagPA in LB broth in the absence of antibiotics showed no structural or segregational plasmid instability over 5 days (~46 cell generations), indicating that the plasmid was stabilised by ORT (Fig. 2). Comparing the mean number of cells (in terms of colony-forming units: cfu) with the plasmid yields prepared from these cultures enabled an estimation of the plasmid copy number of 16 copies per cell.

Expression of PA in Z-ORT/pSCpagPA and Z-pagPA was demonstrated by western blotting (Fig. 3). The strains were grown in minimal medium containing varying concentrations of magnesium to activate the *pagC* promoter controlling expression of the heterologous antigen. As the PA83ec-HlyA fusion is not cleaved following secretion, it is present at a higher molecular mass than the recombinant PA reference. There was a significantly greater amount of PA produced from Z-ORT/pSCpagPA than from Z-pagPA, both in the cell lysate (lanes 5 and 7) and supernatant samples (lanes 11 and 13).

2.2. Colonisation by the Zoosaloral H strains

The *Salmonella* to be used as a live vaccine should retain the capacity to colonise host tissues which was evaluated here in the mouse model. Following oral inoculation of A/Jola mice with $1–5 \times 10^9$ cfu of Z-ORT, Z-ORT/pSCpagPA or Z-pagPA, bacteria could

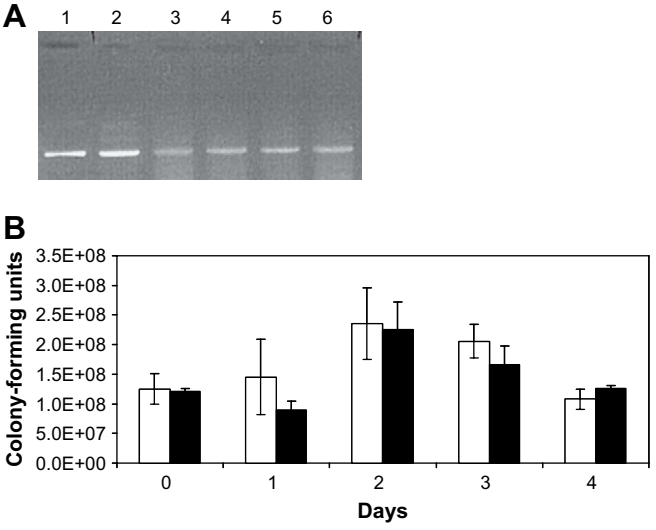

Fig. 2. The serial subculture of Z-ORT/pSCpagPA. (A) An agarose gel showing pSCpagPA plasmid preparations from volumes equivalent to an optical density of $A_{600} = 2.0$. Lane 1: 15 ng pSCpagPA reference DNA; lane 2: inoculum culture (day 0); lanes 3–6: days 1–4. (B) The number of colony-forming units (cfu) from 10^{-7} dilutions plated on LB agar containing 0.5 mM IPTG (white) and LB agar only (black).

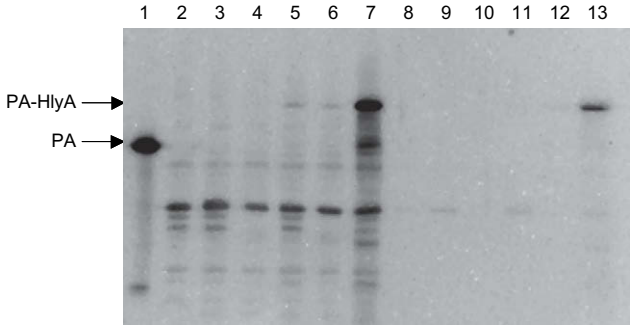

Fig. 3. Analysis of *B. anthracis* PA expression in *S. enterica* serovar Typhimurium strains by western blotting. Cell lysates (lanes 2–7) and culture media (lanes 8–13) were normalised to equivalent cell densities, subjected to SDS-PAGE, western blotted and probed with anti-PA antibody. The *pagC* promoter is repressed by high concentrations of Mg^{2+} and induced in media containing low concentrations of Mg^{2+}. Lane 1: 3.7 ng purified PA; lanes 2 and 8: Zoosaloral H, low [Mg^{2+}]; lanes 3 and 9: Z-ORT, low [Mg^{2+}]; lanes 4 and 10: Z-pagPA, high [Mg^{2+}]; lanes 5 and 11: Z-pagPA, low [Mg^{2+}]; lanes 6 and 12: Z-ORT/pSCpagPA, high [Mg^{2+}]; lanes 7 and 13: Z-ORT/pSCpagPA, low [Mg^{2+}].

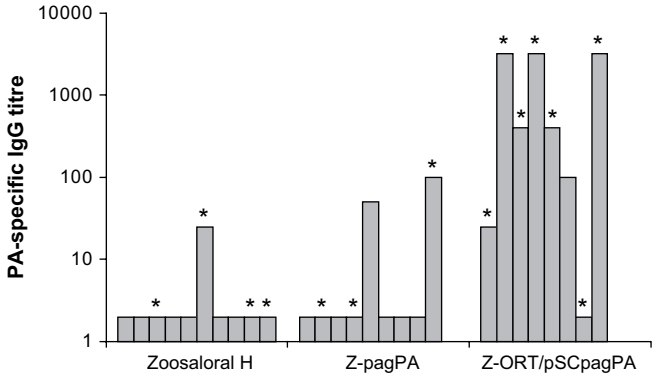

Fig. 4. PA-specific IgG responses induced in individual mice following immunisation with *Salmonella* expressing PA and subsequent boosting with rPA. Groups of A/Jola mice were orally immunised with 3 doses of $1–5 \times 10^8$ cfu of ORT strain Z-ORT/pSCpagPA or chromosomal-integrant strain Z-pagPA and boosted with 5 μg of rPA protein. Blood samples were removed on day 60 and serum IgG responses were measured by ELISA. Results are presented as the reciprocal of the end-point dilution from each animal group. The asterisks indicate mice that survived the *B. anthracis* infection.

be detected in the spleens and Peyer's patches of all groups of mice over a range of time-points post-infection (Table 1). In general, the strains colonised at similar levels, although there was some evidence of lower colonisation by the ORT strain Z-ORT/pSCpagPA in Peyer's patches. Furthermore, similar numbers of Z-ORT/pSCpagPA were detected on LB agar plates with or without IPTG, showing that the pSCpagPA plasmid was stable in the Z-ORT strain *in vivo*; plasmid stability was confirmed by colony PCR (data not shown).

2.3. Immunogenicity and protective efficacy of the Zoosaloral H strains expressing PA

To evaluate the immunogenicity and protection afforded by the ORT *Salmonella* vaccine strains expressing PA, A/Jola mice were orally inoculated with 3 doses of $1–5 \times 10^8$ cfu of Z-ORT/pSCpagPA or the control strain Z-ORT; for comparison, further groups of mice were left uninoculated or inoculated with the chromosomal-integrant strain Z-pagPA. ELISA analysis of mouse sera following the inoculations did not indicate detectable PA-specific serum IgG responses, so the mice were subsequently boosted intra-muscularly (i.m.) on day 42 with unadjuvanted rPA (5 μg in 0.1 ml). This resulted in the boosting of primed PA-specific serum IgG in mice immunised with Z-ORT/pSCpagPA compared to those administered Z-pagPA (Fig. 4). Following challenge with approximately 50 MLD of *B. anthracis* spores, a low level of protection (40%) against *B. anthracis* was provided by the control *Salmonella* strain Zoosaloral H, similar to that provided by the Z-pagPA chromosomal integrant expressing PA. In comparison, mice immunised with the ORT vaccine strain, Z-ORT/pSCpagPA, were afforded significant protection against *B. anthracis* ($p < 0.05$) compared with Zoosaloral H-administered controls (Fig. 5). Although the generally greater PA-specific IgG responses stimulated by Z-ORT/pSCpagPA correlated with a greater level of protection, some mice with undetectable titres were able to survive the *B. anthracis* challenge, indicating that other immune mechanisms are contributing to the protection afforded.

3. Discussion

We have previously demonstrated that ORT is able to effectively stabilise plasmids in *Salmonella*, using both the high copy pUC and low copy pSC101 origins of replication (*ori*) [8]. This work further demonstrates that repressor titration from a plasmid containing the pSC101 *ori*, estimated at 16 copies per cell in *Salmonella*, was sufficient to achieve selection. This estimate of pSCpagPA copy number is similar to that reported for pSC101 of 6–8 copies per chromosome in *E. coli* [16], particularly as an *E. coli* or *Salmonella* culture will contain more than one chromosome per cell on average, and makes it unlikely that compensating mutations have resulted in an increase in copy number. We propose that the low level of basal expression from the *lac* promoter that enables this ORT strain to grow in liquid culture (but not on agar plates) under repressive conditions means that even a relatively small repressor titration from a low copy number plasmid is sufficient to de-repress *lacO*/*P* and enables sufficient *dapD* expression. Plasmid preparations following serial subculture showed structural stability and no change in plasmid copy number.

To demonstrate the efficacy of antibiotic-free ORT maintenance compared to chromosomal integration as a method for gene

Table 1
Colonisation of spleens and Peyer's patches following oral inoculation of A/Jola mice with Zoosaloral H derivatives.

Group	Bacteria (cfu/organ ± SEM)					
	Day 8		Day 12		Day 16	
	S	PP	S	PP	S	PP
Zoosaloral H	67 ± 62	1668 ± 636	283 ± 266	871 ± 356	77 ± 30	1025 ± 326
Z-pagPA	33 ± 25	1050 ± 486	117 ± 60	913 ± 367	187 ± 79	401 ± 211
Z-ORT/pSCpagPA	11 ± 7	387 ± 178*	38 ± 18	330 ± 145	70 ± 60	71 ± 55**

Groups of A/Jola mice were orally inoculated with Z-pagPA, Z-ORT/pSCpagPA or Zoosaloral H. Spleens (S) or 6 Peyer's patches per mouse from 10 mice were removed 8, 12 and 16 days after inoculation and homogenised in PBS before plating onto agar for enumeration of bacteria. *$p < 0.05$ or **$p < 0.005$ compared to Zoosaloral H control.

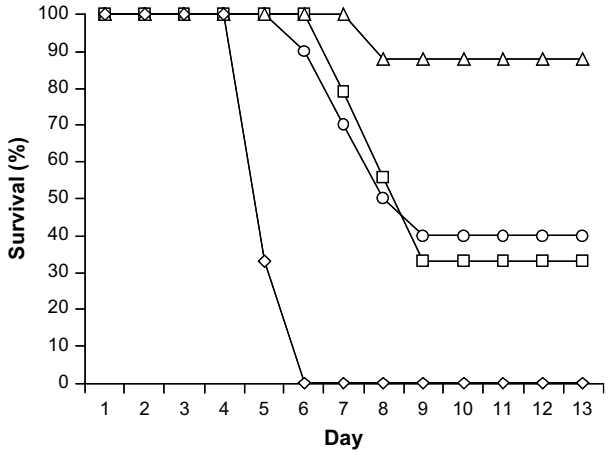

Fig. 5. Survival of immunised mice against *B. anthracis* spores. Groups of A/Jola mice were orally inoculated at 2-week intervals with 3 doses of Z-ORT/pSCpagPA (triangles), Z-pagPA (squares) or Zoosaloral H (circles), and then boosted with 5 μg of rPA i.m. on day 42. Immunised mice and naïve control mice (diamonds) were subsequently challenged i.p. on day 70 with approximately 50 MLD of *B. anthracis* STI spores and observed for 14 days.

stabilisation in live bacterial vector vaccines, we have constructed and compared *Salmonella*-based vaccines expressing the PA protein of *B. anthracis*. We have previously shown that a *S. enterica* serovar Typhimurium Zoosaloral H-based strain (ZpUTKm9.3PA83ec) constitutively expressing and exporting PA from a chromosomally integrated copy of the *PA83ec* gene was able to provide protection against anthrax in mice [17]. However, the integrant was not able to provide protection against anthrax following oral inoculation, perhaps as a result of the metabolic burden placed upon the bacteria during penetration of the gut mucosa. It was later discovered that the T5 promoter in the plasmid used in the same study, pVDL9.3PA83ec, contained a 32 bp deletion that greatly reduced the level of *PA83ec* expression. Indeed, repairing this mutation resulted in a plasmid that could not be replicated in *Salmonella*, presumably due to the detrimental effects of over-expression of *PA83ec-HlyA*. Thus we constructed strains and plasmids to allow *in vivo* regulation of PA expression and to compare an ORT-stabilised plasmid with a chromosomally-integrated antigen gene.

The Z-ORT/pSCpagPA *Salmonella* contains a multi-copy plasmid carrying the PA-encoding gene and other genes required for the haemolysin export system. To minimise the metabolic burden placed upon the *Salmonella* during the infection process, we have constructed recombinants which express these genes under the control of the macrophage-inducible *pagC* promoter, controlled by the PhoP/PhoQ two-component regulatory system which has been found to be an effective *in vivo*-inducible promoter when compared to others [18]. In this study, the *pagC* promoter was shown to stringently control the expression of the PA protein from *Salmonella* under [Mg^{2+}]-limited conditions. *In vitro*, no effect on Z-ORT/pSCpagPA of carrying the multi-copy plasmid could be observed when compared to Zoosaloral H, since both strains grew similarly in broth culture. However, it is possible that there was some effect of carrying the plasmid on colonisation of the *Salmonella in vivo*, since lower numbers of Z-ORT/pSCpagPA than Zoosaloral H were found in the Peyer's patches of infected mice at the two time-points tested, although similar numbers of the strains were observed in the spleens of infected mice, demonstrating an unaffected ability to penetrate deep tissues.

In our experiments, we found that the ORT plasmid allowed a greater level of PA expression than integration into the chromosome of the *Salmonella* vaccine strain, a finding that probably reflects the greater number of PA83ec gene copies in the ORT version. Similarly, the PA-specific IgG responses in mice primed with Z-ORT/pSCpagPA and boosted with rPA were generally greater than those given an equivalent immunisation regimen using Z-pagPA. Furthermore, these findings corresponded with a significantly greater level of protection against *B. anthracis* spores in Z-ORT/pSCpagPA-immunised mice, although the PA-specific IgG response did not correlate with protection in all mice. Similar results were observed in a recent study in which PA was delivered using *Salmonella* [17], and suggest that other cellular immune mechanisms, which were not assayed here, contribute to protection against anthrax. The results in Garmory et al. 2003 [17] demonstrated a higher level of PA-specific IgG from a chromosomal integrant than we measured in this study, but these are different studies using different promoters; the aim of this work is the direct comparison of identical antigen expression cassettes located on a plasmid and in the chromosome. Our results agree with those of Ryan et al. [19], who used live attenuated *Vibrio cholerae* to deliver an antigen consisting of the serine-rich *Entamoeba histolytica* protein fused to the cholera toxin B subunit to mice, and demonstrated that the antigen gene on a multi-copy plasmid elicited a greater immune response than a single chromosomally integrated copy.

To conclude, in this study we have demonstrated the enhanced efficacy of the ORT-stabilised version of a *Salmonella* anthrax vaccine when compared to a similar chromosomal integrant. This finding provides further evidence for the utility of the ORT technology as an antibiotic-free plasmid-maintenance system that may be used effectively for the genetic stabilisation of live bacterial vector vaccines.

4. Material and methods

4.1. Bacterial strains, plasmids and media

All strains were routinely grown in LB medium with additives as required: 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 80 μg/ml DAP, 0.5 mM IPTG, 5% (w/v) sucrose. N-medium was used for evaluating expression from the P_{pagC} promoter: N-medium is composed of N-buffer [20] supplemented with 0.25% (w/v) glucose, 40 μg/ml histidine, 13.5 μg/ml adenine and 100 mM (repression) or 0.1 μM (induction) $MgCl_2$. A His^- Ade^- auxotrophic derivative of *S. enterica* serovar Typhimurium, Zoosaloral H [13], was modified to create an ORT version and for expression of PA. The methylation-deficient *S. enterica* serovar Typhimurium strain LB5010 was used to create an ORT version as an intermediate in the transformation of *Salmonella*. *B. anthracis* STI (Tox^+ Cap^-) was used as the challenge strain in immunised A/Jola mice. The cloning strains of *E. coli* used were DH5α (ATCC number 53868), Top10 (Invitrogen) and DH1*lacdapD* [20].

4.2. Construction of Z-ORT/pSCpagPA

ORT strains of *S. enterica* serovar Typhimurium were generated as described in Garmory et al. 2005 [8]. Zoosaloral H was modified to construct the ORT-VAC strain Z-ORT. In addition, the methylation-deficient strain LB5010 (SGSC number 452) was modified to construct a cloning intermediate, L-ORT, which was designed to overcome the methylation barrier between *E. coli* and *S. enterica* serovar Typhimurium. ORT plasmids were created in *E. coli* DH1*lacdapD* and required passaging through L-ORT prior to transformation into Z-ORT.

First, plasmid pSCbla was made by ligating the origin of replication of pSC101 (DSMZ No. 6202) as a BspHI–XmaI fragment to the

XmaI–AflIII fragment of pORT1 [21], a plasmid encoding the *bla* gene flanked by rare restriction sites and the $lacO_1$ and $lacO_3$ sequences for ORT selection. Plasmid pVDL9.3PA83ec [8] was the source of the *PA83ec* gene (a codon-optimised version of *pagA*) fused to the *hlyABD* export system. This fusion was excised minus the promoter as a BamHI–NotI fragment and cloned into the BamHI–NotI sites of pSCbla to generate pSCPAbla. A *pagC* promoter was amplified by PCR from Zoosaloral H chromosomal DNA using the primers 51pag (5′-GAGCGAACGCGTGCGGCCGCTTAACCACTCT-TAATAATAATG-3′) and 3pag (5′-TACTTCGGATCCGTGATGGT-GATGGTGATGCGATCCTCTCATAACAACTCCTTAATACTAC-3′), then digested using MluI and BamHI, and cloned into the MluI–BamHI sites of pSCPAbla. The *bla* gene was then removed as an FseI fragment, generating the ORT PA expression plasmid pSCpagPA.

4.3. Construction of Z-pagPA

Z-pagPA was engineered by inserting the same *PA83ec-hlyA* cassette as present in pSCpagPA into the chromosome of Zoosaloral H. First, the integration plasmid pLT2fadA was created to target this cassette to a chromosomal space between the *fadA* and *STM3981* genes using Zoosaloral H chromosomal DNA as the PCR template. Briefly, the STM3981 locus was amplified using primers 5STM (5′-GAGCGACATATGCCCAGCGATGGCCGCGGCTTAGAACA-3′) and 3STM (5′-GAGCGAAGCTTCGAATCCCCGGGCACATAGA-3′), and digested using NdeI and HindIII. The *fadA* locus was amplified using primers 5fad (5′-GAGCGAAGCTTGAGCTTGAGCGGCGGCCGCGAGCGCT-TAAGCATAAGTTATCTATGTGCCCG-3′) and 3fad (5′-GAGCGGG ATCCCCCAGCATCCGTCAATTATGGGTTAC-3′), and digested using BamHI and HindIII. Both digested PCR products were then ligated to pUC18 cut with BamHI and NdeI in a three-fragment ligation to create pLT2fadA. pLT2dif was then constructed by ligating the HindIII–NotI fragment of pTOPO–DifCAT [15], encoding the *cat* gene flanked by *dif* sites, into pLT2fadA cut with the same enzymes. The NotI fragment of pSCpagPA was then ligated into pLT2dif to create pLT2pagPA. Finally, a BseYI fragment of pLT2pagPA was transformed into Zoosaloral H, generating Z-pagPA after Xer-mediated deletion of the *cat* gene ('Xer-cise') [15].

4.4. Detection of PA expression

Cells were inoculated from single colonies into 5 ml LB broth and cultured overnight (37 °C, 200 rpm). This culture was used to inoculate 50 ml N-medium (with IPTG for Z-ORT) containing inducive or repressive magnesium concentrations, in 250 ml baffled Erlenmeyer flasks, to an initial optical density of $A_{600\ nm} = 0.01$ and incubated at 37 °C, 200 rpm for 48 h. Cell numbers in these experiments were normalised to $A_{600\ nm}$ units, representing the same number of cells as present in a culture with an optical density of $A_{600\ nm} = 1.0$. Culture volumes of 0.5 $A_{600\ nm}$ units were centrifuged and pellets re-suspended in 50 mM Tris–HCl (pH 8.0) and 10 mM $MgCl_2$ and incubated on ice for 2 h with Benzonase® (Merck). Samples of the cell lysates and supernatants were then mixed with reducing agent and sample loading buffer, and heated for 10 min at 70 °C prior to loading of 5.6×10^{-6} $A_{600\ nm}$ units in each lane of a 4–12% (w/v) SDS-polyacrylamide gel. Following electrophoresis in MOPS buffer, the gel was transferred to a nitrocellulose membrane, probed with the PA-specific antibody C3 (produced at the Defence Science and Technology Laboratory), then with an HRP-labelled rabbit anti-mouse antibody (Sigma A-9044). The bound antibody was detected following treatment with peroxidase substrate (Sigma CPS-1) by chemiluminescence using an AlphaImager (Alpha Innotech).

4.5. Growth analysis, plasmid maintenance and copy number

For growth analysis, single colonies of the three strains under investigation were inoculated into 5 ml LB broth in 30 ml tubes and incubated overnight at 37 °C, 200 rpm. These pre-cultures were used to inoculate 50 ml LB broth in 250 ml baffled Erlenmeyer flasks to an initial optical density of $A_{600\ nm} = 0.1$ and readings were taken hourly.

To determine plasmid stability, 5 ml pre-cultures were prepared as described above. The optical density was determined and flasks containing 50 ml fresh LB broth were inoculated to $A_{600\ nm} = 0.001$ and incubated for 24 h. The process was repeated for 4 more days. Samples of 2.0 $A_{600\ nm}$ units were taken daily, the plasmids were extracted using the Qiaprep kit (Qiagen) and examined by agarose gel electrophoresis (Fig. 2A). Additionally, 10^{-7} $A_{600\ nm}$ units were plated onto permissive and non-permissive media, and the colonies counted to determine the proportion of cells retaining the pSCpagPA plasmid (Fig. 2B).

The plating following each day of the subculture allowed the mean culture densities from which the plasmids were prepared to be calculated as 1.5×10^8 cfu per $A_{600\ nm}$ unit (Fig. 2A). Plasmids had therefore been prepared from 3×10^8 cfu (2.0 $A_{600\ nm}$ units, as described above) and 18% of each preparation (i.e. the plasmid DNA generated from 5.4×10^7 cfu) loaded per lane of the gel on days 1–4, giving a mass of 10 ng DNA per lane (confirmed by densitometry) relative to the reference DNA. The molecular mass of pSCpagPA is 7 MDa (10596 bp × 660 Da/bp), with 10 ng being 1.43 amol (10^{-8}/7×10^6). Therefore an estimate of plasmid copy number is $1.43 \times 10^{-15} \times 6.022 \times 10^{23} / 5.4 \times 10^7 = 15.9$ plasmid copies per cell.

4.6. Inoculations and colonisation

Strains were cultured statically overnight at 37 °C, then centrifuged (6000 *g*, 20 min, 4 °C), washed once in phosphate-buffered saline (PBS), re-centrifuged and re-suspended in PBS to a final cell density of approximately $1–5 \times 10^{10}$ cfu/ml (for colonisation studies) or $1–5 \times 10^9$ cfu/ml (for protection studies). Viable bacteria were enumerated on LB agar plates. Subsequently, groups of 30 female 8–12-week old A/Jola mice (Charles River Laboratories) were orally inoculated with approximately $1–5 \times 10^9$ cfu/100 μl dose of *Salmonella* by gavage. On days 8, 12 and 16 post-inoculation, 10 mice were culled by cervical dislocation and for each mouse the spleen and 6 Peyer's patches (PP) were removed. The spleen and six PP were homogenised in 1 ml PBS using 50 μm cell strainers (Becton Dickinson Labware) and suitable dilutions were plated onto LB agar for enumeration.

4.7. Immune response analysis and B. anthracis challenge

Groups of 8–10 female A/Jola mice were orally inoculated with approximately $1–5 \times 10^8$ cfu/100 μl of the relevant *Salmonella* strain on days 0, 14 and 28. The mice were individually microchipped with a unique reference number so that their immune responses could be followed throughout the experiment. On day 39, tail vein blood samples were taken from the mice and used in ELISA to detect the presence of serum IgG against PA. On day 42, PA-specific immune responses were boosted by intramuscular injection with 5 μg rPA in 100 μl PBS and subsequently, on day 60, further tail vein samples were taken from the mice for ELISA analysis. ELISAs were carried out as described previously [17] and all samples were assayed in duplicate. End-point antibody titres were expressed as the maximum dilution of sample giving an absorbance of greater than 0.1 $A_{414\ nm}$ units after subtraction of the absorbance due to non-specific binding detected in control sera from mice immunised with the control strain Z-ORT. The ELISA results are presented as the $\log_{10}$

of the reciprocal of the dilution, and the mean of duplicate values for each sample. On day 70 the mice were challenged intra-peritoneally (i.p.) with approximately 5×10^4 *B. anthracis* STI spores prepared as described previously [22] in 0.1 ml PBS. This is equivalent to approximately 50 median lethal doses (MLD), based upon a previous study carried out with the same strain of *B. anthracis*, the same mouse strain and a similar experimental procedure [23]. The mice were observed for 14 days with strict observance of humane end-points and the time to death was recorded.

4.8. Statistical analyses

Values for the mean and standard error of the mean were calculated. The statistical significance of survival was analysed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-analysis test using the GraphPad Prism version 3.02 for Windows (GraphPad Software). The statistical significance of immune responses was analysed using Student's *t*-test and also using GraphPad Prism. Probability values (p) of ≤ 0.05 were taken as significant.

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