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Evaluation of the humoral immune response in mice orally vaccinated with live recombinant attenuated *Salmonella enterica* delivering a secreted form of *Yersinia pestis* PsaA

Ascención Torres-Escobar, María Dolores Juárez-Rodríguez, Christine G. Branger, and Roy Curtiss III

Center for Infectious Disease and Vaccinology at the Biodesign Institute and School of Life Sciences, Arizona State University. Tempe, AZ, 85287-5401, USA

Abstract

Yersinia pestis PsaA is an adhesin that is synthesized inside macrophages. Here, we evaluated the immune profile of codon-optimized *Y. pestis* PsaA synthesized in a live recombinant attenuated *Salmonella* vaccine (RASV) strain χ 9558. Oral immunization of BALB/c mice with χ 9558(pYA3705) delivering a secreted form of PsaA, elicited a systemic PsaA-specific immunoglobulin G (IgG) response but offered limited protection against lethal challenge with the intranasally introduced *Y. pestis* CO92 strain. Our results suggest that appropriate fine-tuning of *Y. pestis* PsaA delivery by RASV could improve its protective role in curtailing plague colonization and infection.

Keywords

Asd⁺; vaccine plasmid; codon-optimized; PsaB chaperone protein; PsaC usher protein; PsaA antigen

1. Introduction

Plague is an often fulminant systemic zoonosis caused by *Y. pestis*, a Gram-negative facultative bacterium [1]. This disease is transmitted from rodents to humans by the bite of an infected flea, which usually causes the bubonic form of plague [2]. Septicemic plague can occur if *Y. pestis*-infected material enters the body through damaged skin and multiplies within the bloodstream, which may also occur as a complication of bubonic plague. Plague remains a serious problem in public health. Its wide-spread aerosol dissemination when an infected person coughs or sneezes (pneumonic plague) and its high mortality rate when left untreated, makes *Y. pestis* an especially dangerous human pathogen and antibiotic-resistant strains have recently been discovered [3,4]. At present, there is no commercially available vaccine against the plague [5,6]. Therefore, the development of a safe and effective vaccine is desirable. *Y. pestis* antigens that are exposed directly to the mucosal surface are good potential candidates for the development of such vaccines [7]. The PsaA protein encoded by

Corresponding Author: Roy Curtiss III, The Biodesign Institute, Center for Infectious Diseases and Vaccinology, Arizona State University, PO Box 875401, 1001 S. McAllister Avenue, Tempe, AZ 85287-5401. Tel 480-727-0445. Fax 480-727-0466. Tempe, Arizona 85287. rcurtiss@asu.edu.

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Y. pestis has a molecular mass of 15 kDa and forms fibrillar organelles that are exposed on the cell surface as a single strand, multistranded bundles or large aggregates [8]. The expression of *psaA* has been detected in all three clinical forms of plague (bubonic, septicemic and pneumonic) [9,10,11]. The *psaA* gene is expressed in cells grown in acidic culture media (pH 5–6.8) containing low Mg^{+2} , limited nutrients, high salt (e.g., hyperosmotic stress conditions) from 35°C to 41°C [12,13,14]

Y. pestis *psaA* is encoded along with other four genes in the *psaEFABC* operon. The *psaE*, *psaF*, *psaB* and *psaC* genes have significant homologies to gene families encoding activator, sensor, chaperone and usher proteins, respectively [8,13,15]. The global regulator RovA and the quorum sensing system *ypeR/I*, *yspR/I* activate positively *psaA* expression [9,16], while the Fur protein acts as a repressor under iron-rich conditions [17].

Y. pestis that synthesizes PsaA is more rapidly fatal to mice than bacteria that do not produce this antigen [12]. The lack of PsaA synthesis in the KIM5 strain reduces its virulence and increases its LD₅₀ at least 100 fold, in retroorbitally injected mice [13]. However, *psaA* mutations in the *Y. pestis* 231 and I-996 strains do not alter virulence in mice subcutaneously inoculated [18].

Y. pestis PsaA is able to reduce phagocytosis by macrophages [19,20]. However, the molecular mechanism of PsaA-mediated anti-phagocytosis has not been elucidated. In addition, PsaA mediates erythrocyte agglutination in a wide variety of species [12,21]. Purified PsaA binds to several subclasses of human immunoglobulin G (IgG) by acting as a bacterial Fc receptor [22], PsaA can also act as an adhesin for respiratory tract epithelial cells by binding to glycosphingolipids or phosphatidylcholine lipid receptors [23,24].

The antibody profile of rabbits immunized with a live EV76 vaccine strain showed high levels of anti-PsaA (IgG) at 42 days after immunization [25]. It was recently shown that PsaA and PsaB induce a strong T-cell response in mice immunized with the EV76 strain [26]. Similarly, mice immunized with 40 µg of *Y. pestis* PsaA along with an aluminum salt adjuvant (alhydrogel,) exhibited a strong humoral immune response and a significant protection (70%) against a pneumonic plague intranasal infection with the strain KIM5 in the iron dextran-treated mouse model [27]. Together, these results confirm that PsaA is a good potential protein target for immunization against plague.

Live attenuated *Salmonella* has been developed as an oral homologous vaccine and a carrier of heterologous antigens due to its ability to stimulate the mucosa for effective antigen delivery [28,29]. Several different promoter sequences can be used to drive the expression of genes encoding potential protective antigens of interest [30,31,32,33]. Numerous approaches have been employed to delete *Salmonella* genes that encode enzymes involved in the biosynthesis of aromatic compounds to confer attenuation and essential components of the peptidoglycan layer of the bacterial cell wall, allowing the use of plasmid systems that can be maintained without the use of antibiotic-resistance markers. Replication plasmids with different copy-numbers have also been used to improve plasmid stability and to attain a better balance between plasmid replication and the synthesis of heterologous protective antigens [34,35,36]. The goal of these modifications has been to improve the ability of *Salmonella* to survive in the gastro-intestinal tract, and thus its ability to reach the inductive lymphoid tissues, where it can elicit mucosal and systemic immune responses.

Here, we evaluate the immune response elicited by *Y. pestis* PsaA encoded in the Asd⁺ vaccine vector pYA3705 [15] and its efficacy as a protective antigen against a lethal *Y. pestis* challenge. We used the new generation RASV strain χ 9558, which harbors deletion/insertion mutations that modify metabolic functions and virulence attributes to allow

regulated delayed attenuation [37,38]. This strain has been shown to be both safe [39] and immunogenic in adult and infant mice [40,41].

2. Materials and Methods

2.1 Bacterial strains, media and growth conditions

The *Salmonella enterica* serovar Typhimurium χ 9558 strain $\Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{crp527}::TT araC P_{BAD} crp \Delta asd27::TT araC P_{BAD} c2 \Delta araE25 \Delta araBAD23 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta agfBAC811$ was grown at 37°C in LB broth [42] or on LB agar (1.5%) or on MacConkey agar (DIFCO). The medium was supplemented with 0.2% mannose and 0.2% arabinose. Diaminopimelic acid (DAP) was added at 50 μ g/ml when required for growth of non-complemented Δasd strains [35].

2.2 Construction of the vaccine vector pYA3705

The antibiotic-resistance free vaccine plasmid pYA3705 expressing codon-optimized *Y. pestis* *psaA* [15] was derived from pYA3342 (pBR *ori*) [43]. The pYA3705 contains *Y. pestis* *psaA* with 15 codons substituted with the most frequently used *Salmonella* codons to optimize its expression in RASV strains and also contains the *Y. pestis* *psaB* and *psaC* (encoding chaperone and usher proteins, respectively) (Fig. 1A), which are essential for translocation of PsaA to the surface of *Salmonella* [8,13,15].

2.3 Expression of *psaA* from pYA3705

One colony of the RASV χ 9558 strain harboring pYA3342 or pYA3705 was grown in 3 ml of LB supplemented with 0.2% mannose and 0.2% arabinose with shaking at 37°C to an optical density at 600nm (OD_{600nm}) of 0.8 units.

2.4 Cell fractionation

Cell fractions were prepared from 1 ml of culture using the PeriPreps Periplasting Kit (Epicentre) according to the manufacturer's instructions. To isolate proteins released into the culture supernatants, 1 ml of culture cells was pelleted by centrifugation and the supernatant was collected, filtered (0.22- μ m pore size, Corning) and precipitated with 10% TCA. The pellet obtained by centrifugation was then resuspended in 100 μ l of lithium dodecyl sulfate sample buffer (LDS) (Invitrogen). For total extract, 10 μ l of 1 ml of culture was taken. For each fraction, 10 μ l were mixed with 10 μ l of LDS sample buffer, boiled for 5–10 minutes, separated by SDS-PAGE (NuPAGE 4–12% Bis-Tris, Invitrogen) and immunoblotted. The recombinant protein was identified with rabbit anti-PsaA serum (1:15,000) [15] followed by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). Mouse anti- σ^{70} monoclonal antibody (1:1000) was used as a loading marker (Neoclone). All experiments were performed in triplicate.

2.5 Antigen preparation

The expression of the recombinant PsaA-AU1-6 $\times$ His protein from plasmid pYA3883 has been described previously [15]. The protein was purified by nickel-nitrilotriacetic acid agarose chromatography under native conditions. Eluted fractions containing the purified PsaA-AU1-6 $\times$ His were identified by SDS-PAGE, pooled, dialyzed against phosphate-buffered saline (PBS) and then concentrated. Protein concentration was determined by a Bradford assay using bovine serum albumin as a standard. Endotoxins were removed with a Detoxi-Gel endotoxin removing gel (Pierce, Rockford, IL). The purified PsaA-AU1-6 $\times$ His was subsequently used for ELISA assays. *Salmonella* outer membrane proteins (SOMPs)

were obtained using sonication and the Triton X-100 extraction procedure described previously [44].

2.6 Immunization of mice

Female BALB/c mice (6–7 weeks old) were purchased from Charles River Laboratories. Mice were acclimated for 7 days before starting the experiments. Mice were deprived of food and water for 6 h before oral immunization. The RASV χ 9558 harboring pYA3342 or pYA3705 was grown for 18 h statically in 5 ml LB with 0.2% mannose and 0.2% arabinose at 37°C. These cultures were then used to inoculate 100 ml of LB with 0.2% mannose and 0.2% arabinose, which were grown at 37°C with shaking (180 rpm) to an OD_{600nm} of 0.8 units. Cells were pelleted by centrifugation at room temperature (4,000 × g for 15 min) and the pellet was resuspended in 1 ml of buffered saline containing 0.01% gelatin (BSG) [45]. Dilutions of the vaccine strains were plated onto LB agar (0.2% arabinose, 0.2% mannose) to determine bacterial titers. Mice were divided into a group of 40 mice (for vaccination with RASV χ 9558 harboring pYA3342), another group of 40 mice (for vaccination with RASV χ 9558 harboring pYA3705) and a third group of 24 mice (for vaccination with BSG alone) and orally inoculated with 20 μ l of respective RASV suspended in BSG containing 2×10^9 colony forming units (CFU) or BSG alone on days 0 and 9. Water and food were returned to the mice 30 minutes after immunization. Blood samples were obtained by sub-mandibular vein puncture 2 days before vaccination and on days 22 and 32 after the first vaccination. Next, the blood was incubated at 37°C for 60 min and centrifuged at 4,000 × g for 5 min. The serum was removed and stored at –70°C. Vaginal secretion specimens were collected two days before vaccination and at days 22 and 32 after the first vaccination in a 50 μ l BSG wash and stored at –20°C. The Arizona State University Animal Care and Use Committee approved all the animal procedures.

2.7 Challenge experiments

To assess the protective effects of RASV against *Y. pestis* in immunized mice, groups of 8 immunized or control mice were challenged with *Y. pestis* strain CO92 (biovar Orientalis) [46] at 28 days after the second immunization (day 35). For subcutaneous challenge (s.c.), mice were subcutaneously inoculated with a low dose (LD) of 4.49×10^2 CFU or a high dose (HD) of 5.63×10^3 CFU of *Y. pestis* grown at 28°C in heart infusion broth, HIB (DIFCO) supplemented with 0.2% xylose. For intranasal challenge, mice were anesthetized with a mixture of 0.5% ketamine and 0.01% xylazine administered intramuscularly and 25 μ l PBS containing a LD of 4.1×10^3 CFU or a HD of 4.4×10^4 CFU of *Y. pestis* grown in HIB at 37°C supplemented with 0.2% xylose and 2 mM CaCl₂ was inoculated intranasally to each group of mice. The mice were observed daily and mortality was recorded until 14 days after the challenge. The surviving animals were euthanized at the end of the experiment and blood samples were obtained by intra-cardiac puncture. The blood samples were left overnight at 4°C and then centrifuged at 4,000 × g for 5 min. The sera were removed and half of each sample was incubated for three days on tryptose blood agar (TBA) plates at 28°C to verify that they were free of bacteria. The serum samples were later used for serological analyses.

2.8 Enzyme-linked immunosorbent assay (ELISA)

IgG, IgG2a, IgG1 and IgA antibody titers against PsaA and SOMP were determined by ELISA. Briefly, Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc Rochester, NY, USA) were coated with purified PsaA-AU1-6×His (1 μ g/well) or SOMP (500 ng/well) suspended in 0.05 M carbonate-bicarbonate buffer, pH 9.6. The coated plates were incubated overnight at 4°C and then washed three times with PBS containing 0.05% Tween 20. Free binding sites were blocked with PBS-0.1% Tween 20 containing 2% BSA. Vaginal washes and sera obtained from the same experimental group were pooled. The serum pools

were diluted by two-fold serial dilutions from an initial dilution of 1:200 in PBS. The vaginal secretion pools were serially diluted by two-fold serial dilutions from an initial dilution of 1:10 in PBS. Aliquots of 100 μ l volume of serially diluted samples were added to triplicate wells and incubated overnight at 4°C. Plates were washed and treated with HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA (1:4,000 dilution, Southern Biotechnology Inc., Birmingham, AL). Wells were developed with 200 μ /well *o*-phenylenediamine dihydrochloride (OPD) at 0.4 mg/ml in phosphate-citrate buffer containing H₂O₂ (Sigma). Color development was stopped by the addition of 50 μ l 3M H₂SO₄ per 200 μ l reaction solution. Absorbance was recorded at 492 nm using an automated ELISA plate reader (LabSystems Multiskan MCC/340). Endpoint titers were expressed as the reciprocal log₂ of the last sample dilution giving an absorbance of 0.1 OD units above negative controls after a 1.5 h of incubation.

2.9 Statistical analysis

Differences in antibody responses between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Differences with $P < 0.05$ were considered significant at the 95% confidence interval. Statistical analysis was performed in GraphPad Prism Software (GraphPad Software, San Diego, CA). The Kaplan-Meier method (GraphPad Prism Software) was applied to obtain the survival fractions following s.c. or i.n. *Y. pestis* challenges of orally immunized mice.

3. Results

3.1 Expression and stability of PsaA in RASV

The AsdA⁺ recombinant plasmid pYA3705 containing the *psaABC* operon under the control of the LacI regulatable P_{trc} was constructed (Figure 1A). The PsaA synthesized by *Salmonella* χ 9558(pYA3705) is delivered to the bacterial surface through its own PsaB/PsaC secretion system. The generation of strain χ 9558 has been described in detail by Gunn et al. [37]. It should be noted that χ 9558 contains the deletion-insertion mutation Δ relA198::*araC* P_{BAD} *lacI* TT (Transcription Terminator). LacI is a repressor protein whose synthesis is regulated by the arabinose concentration used to achieve regulated delayed synthesis of PsaA encoded by *psaA* that is under transcriptional control of P_{trc}. This is important as it prevents the expression of *psaABC* during vaccination, which could reduce colonization ability and thus the immunogenicity of the RASV. Analysis of the expression profile of χ 9558(pYA3705) showed proteins with molecular masses of 15 kDa and 18 kDa in the total extract and 15 kDa in the supernatant, that reacted specifically with the anti-PsaA polyclonal antibody. The expression profile is proportional to the concentration of arabinose in the medium (Fig. 1B). The plasmid was maintained and protein synthesis was stable when the strain was grown under nonselective conditions in the presence of DAP for 50 generations (data not shown) [15, 43].

3.2 IgG antibody responses to PsaA

To investigate the immunogenicity of the PsaA protein delivered by the RASV strain, we orally immunized groups of BALB/c mice on days 0 and 9 with *S. Typhimurium* χ 9558 harboring either the control vector (pYA3342) or a vaccine vector that specified synthesis of PsaA (pYA3705). Serum IgG responses to PsaA and SOMPs from immunized mice were measured by ELISA. Systemic IgG responses to PsaA were observed at 2 weeks post immunization and increased over time. The PsaA vaccinated group had significantly higher total anti-PsaA IgG titers on day 32 ($P < 0.05$) than did mice immunized with the vector control strain χ 9558(pYA3342) or in preimmune serum (Fig 2A). These results indicate that PsaA delivered by χ 9558(pYA3705) induces systemic antibody IgG titers against PsaA. In contrast, the total IgG to SOMPs was significantly higher in mice immunized with

χ 9558(pYA3342) at day 22 and day 32 ($P < 0.001$), and lower levels of IgG to SOMPs were detected in χ 9558(pYA3705) immunized mice (Fig. 2B).

3.3 IgG antibody subclass responses to PsaA

The immune response induced by χ 9558(pYA3705) in mice was further examined by ELISA analysis of IgG2a and IgG1 levels in preimmune serum and on days 22 and 32 after primary immunization. In mice, IgG1 is associated with a Th2-like response, while a Th1 response is associated with the induction of IgG2a [47]. The IgG2a and IgG1 titers were only increased to PsaA after immunization on day 22 and increased significantly on day 32 ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 2C). Titers of IgG2a were similar to those of IgG1 in vaccinated mice. This data indicates that the RASV synthesizing PsaA induced both Th2-related IgG1 and Th1-related IgG2a antibody responses.

3.4 Mucosal IgA responses to PsaA

IgA antibodies are the principal mucosal antibody class produced by local plasma cells and are the first line of immune defense against infection [48]. Moderate secretory IgA titers against PsaA were detected in vaginal fluids from mice immunized with *Salmonella* expressing PsaA in comparison to immunization with the vector control strain χ 9558(pYA3342) or in preimmune serum (Fig. 2D).

3.5 Evaluation of protective immunity

To test the protective efficacy of χ 9558 synthesizing PsaA from pYA3705, BALB/c mice were orally immunized with χ 9558(pYA3342), χ 9558(pYA3705) or with BSG on days 0 and 9 as described above. At 28 days after the second immunization, the mice were challenged intranasally with 4.4×10^4 CFU (HD) or with 4.1×10^3 CFU (LD) of *Y. pestis* CO92. Another group of mice was challenged subcutaneously with 5.63×10^3 CFU (HD) or with 4.49×10^2 CFU (LD). All the mice immunized with *Salmonella* χ 9558(pYA3342) (control group) or χ 9558(pYA3705) synthesizing PsaA died between days 3 and 9 after subcutaneous challenge with the HD or LD of virulent *Y. pestis* (Fig. 3A). Two of eight immunized mice with RASV χ 9558(pYA3705) strain survived intranasal challenge with the LD of virulent *Y. pestis* and one of eight mice survived intranasal challenge with the HD (Fig. 3B).

3.6 IgG antibody responses to PsaA and SOMPs in surviving mice after intranasal *Y. pestis* challenge

The serum immune response to PsaA was examined by measuring the levels of total IgG isotype and subclasses IgG1 and IgG2a in surviving mice after intranasal challenge. The level of total IgG to PsaA in mice surviving intranasal challenge was higher than in orally immunized mice on days 22 and 32, and significantly higher in comparison with the preimmune serum for both the LD ($P < 0.01$) and HD ($P < 0.001$) challenges (Fig. 4A).

Titers of IgG2a were slightly high to those of IgG1 in LD post-challenged mice, but the ratio of IgG2a/IgG1 was more evident in the surviving mice challenged with HD ($P < 0.001$) (Fig. 4B). The anti-SOMP IgG levels were higher in surviving mice than the titers observed in mice immunized with χ 9558(pYA3705) on days 22 and 32. Regardless of whether the mice were challenged intranasally with a LD or HD of *Y. pestis*, the SOMP IgG levels were similar among the surviving mice (Fig. 4C).

4. Discussion

PsaA is an immunogenic *Y. pestis* surface protein that induces high-titer antibodies in rabbits immunized with the live attenuated *Y. pestis* vaccine strain EV76 [25]. Moreover, PsaA and

PsaB induce a strong T-cell response [27], but mice immunized with 15 µg of either protein along with the adjuvant CpG1826-ODN were not protected from subcutaneous challenge with the lethal *Y. pestis* 201 strain [26]. In contrast, immunization with 40 µg of recombinant *Y. pestis* PsaA along with the aluminum salt adjuvant alhydrogel elicited a strong humoral immune response and significant protection (70%) against a pneumonic plague intranasal infection with the strain KIM5 in the iron dextran-treated mouse model [27].

In this study, the expression of *Y. pestis* *psaA* was under the control of P_{trc} for robust synthesis and delivery by *Salmonella* Typhimurium strain χ 9558(pYA3705) that also synthesized the PsaA secretory system (PsaB/PsaC). PsaA synthesis was modulated by a chromosomal insertion of *araC* P_{BAD} controlling the synthesis of LacI, which repressed the transcription of P_{trc} -*psaA* from pYA3705 in the presence of 0.2% arabinose. When transferred to an arabinose-free environment (i.e., host tissues when *Salmonella* colonizes and invades into mucosal and internal lymphoid tissues), the remaining intracellular arabinose in the bacteria is eventually exhausted and the synthesis of PsaA can be initiated in the inductive lymphoid tissues, eliciting immunological responses. The new regulated/delayed/attenuated strain χ 9558 was evaluated for entry into the brain of mice after oral, intranasal or intraperitoneal introduction and shown to be safe [39]. Moreover, this strain performed comparably to other live attenuated *Salmonella* strains in an immunization assay involving a secreted form of the alpha-helical region of pneumococcal surface protein A (PspA); it was found to elicit PspA-specific antibody responses and specific cytokine secretion in vaccinated mice resulting in significant protection against pneumococcal challenge. More recently χ 9558 was also evaluated in infant mice with similar results [37,40,41].

Here, we observed a moderate increase in the level of PsaA-specific IgA in the vaginal fluids of mice immunized with χ 9558(pYA3705) synthesizing *Y. pestis* PsaA. Live *Salmonella* are especially useful since they induce strong and sustained humoral as well as cellular immune response both in the mucosal and systemic compartments. Distinct anatomical and functional features characterize the mucosal immune system as in the epithelia of the gut, airways and upper genital tract. Thus, the stimulation by the antigens at mucosal surfaces such as the IgA antibodies produced by plasma cells may determine the efficacy of vaccine strategies [49]. It should be emphasized that it has been known for 15 years that delivery of a recombinant *Salmonella* vaccine by oral, intranasal, vaginal, rectal, intravenous and intraperitoneal routes stimulates mucosal immunity with production of secretory IgA in all mucosal tissues and from all secretory glands [50,51]. It is the only vaccine strategy that can do this as well as being able to induce systemic antibody and cellular immunities. If the moderated level of IgA elicited by oral immunization with χ 9558(pYA3705) offers protection against pulmonary *Y. pestis* infection detected in this study will require further specific experiments.

We also observed a significant immune response represented by the increase of PsaA-specific serum IgG antibody levels in immunized mice. The ratio of IgG2a/IgG1 subtypes was similar in vaccinated mice and in mice after LD challenge. A lower induction of anti-SOMP response was observed in χ 9558(pYA3705) immunized mice compared with mice receiving χ 9558(pYA3342) (control vector); this might be due to an over-synthesis of PsaA which could lead to cause a metabolic burden resulting in the low levels of anti-SOMP IgG.

Although significant levels of anti-PsaA serum IgG were induced, we found that delivery of PsaA by χ 9558(pYA3705) allowed 12.5% and 25% mice to survive intranasal HD and LD challenge, respectively. However, all the mice challenged by the subcutaneous route succumbed between day 3 and day 9 post-challenge with either dose. In contrast, parallel studies using the *Y. pestis* LcrV196 antigen and the same schedule of immunization and

challenge with LD and HD of *Y. pestis* showed that mice orally immunized with χ 9447 expressing optimized *lcrV196* from pYA4535 (AsdA⁺ runaway derivative) exhibited protective immunity against both intranasal and subcutaneous challenges with *Y. pestis* CO92 strain [52]. Interestingly, the serum of surviving mice showed an increase in IgG2a and a decrease in IgG1 in comparison with χ 9558(pYA3705) immunized mice, which suggests that surviving mice had a slightly bias toward a Th1 response. The PsaA-specific IgG2a antibody may facilitate the phagocytosis of the pathogen by opsonization, among other mechanisms. Mice surviving challenge also showed a higher titer of anti-SOMP IgG than χ 9558(pYA3705)-immunized mice, but this titer was low when compared to that of control (χ 9558(pYA3342)-immunized mice). These results may suggest that the protective response to intranasal challenge could be mediated by the systemic induction of anti-PsaA IgG with a concomitant increase in the IgG2a/IgG1 ratio generated in mice orally immunized with RASV χ 9558(pYA3705). Future experiments will be required to validate this observation.

On the basis of our results, the humoral immunity to PsaA tested, was not sufficient to afford protection against *Y. pestis* infection introduced by the s.c. route. This evidence is in agreement with other studies in which PsaA was non-protective against bubonic *Y. pestis* infection [26,53]. Moreover, our result is also in accordance with a preliminary report, which indicated that purified PsaA immunization in the presence of the adjuvant alhydrogel offers protection against pulmonary *Y. pestis* infection [27]. Transcriptional profiling of *Y. pestis* in mice infected by the intranasal route revealed that *psaA* was upregulated and highly persistent in the mouse lung from 4 to 48 hrs [54]. In addition, PsaA has been shown to have antiphagocytic properties with murine macrophages, reduces Yop translocation to HEp-2 and THP-1 cells and blocks bacterial uptake by binding to a host receptor [19,55,56]. However, little is known about the interaction of *Y. pestis* with major respiratory tract cells and whether PsaA plays a role in pneumonic plague. If the PsaA antigen can be neutralized effectively by antibodies and thus block pulmonary *Y. pestis* infection will require more extensive studies.

In spite of the limited protective response generated against lethal *Y. pestis* challenge, it is reasonable to propose further studies aimed at preventing an antigen burden effect by using *psaA*-encoding plasmids with a low copy number replication, such as p15A or very-low copy number, such as pSC101. Additionally, further modifications of the *Salmonella* vaccine strain might enhance its immunogenicity and increase the efficacy of PsaA secretion [15]. As shown by the results of this study, PsaA delivered by attenuated *Salmonella* is a promising component to include for the development of a vaccine against *Y. pestis*.

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Figure 1.

A. Map of the pYA3705 plasmid. B. Immunoblot of total extract and supernatant of the *S. enterica* serovar Typhimurium χ 9558 harboring pYA3705 with a PsaA-specific polyclonal rabbit antibody. The χ 9558 strain was grown in LB with 0.2% mannose (+) and 0.2% arabinose (+) or without arabinose at 37°C, with shaking to an OD_{600nm} of 0.8. C. Immunoblot of an analogous total extract and supernatant of χ 9558 harboring pYA3705 blotted with anti- σ^{70} antibody. The σ^{70} was used as a cytoplasmic marker and as a loading control.

Figure 2.

Humoral immune responses to PsaA and SOMPs in mice orally immunized with *Salmonella* strain χ 9558(pYA3705) (PsaA) and χ 9558(pYA3342) (control) measured by ELISA. A. Serum IgG total response to PsaA. B. *S. Typhimurium* anti-SOMP IgG. C. Subclasses (gG1 and IgG2a) of anti-PsaA serum. D. Mucosal IgA response to PsaA in vaginal secretions. The data represent the endpoint of antibodies in pooled pre-immune serum (PI) and in immunized mouse serum at 22 and 32 days after the first immunization. Error bars represent variations between triplicate wells. The statistical significance was calculated by one-way ANOVA and Tukey's post-test (*P < 0.05; **P < 0.01; *** < 0.001).

Figure 3.

Survival percentage of immunized BALB/c mice after *Y. pestis* challenge. Mice were orally immunized on days 0 and 9 with χ 9558(pYA3342) (vector control), χ 9558(pYA3705) (synthesizing PsaA) or BSG (control). A. Mice were subcutaneously (s.c.) challenged with 4.49×10^2 CFU (LD) or 5.63×10^3 CFU (HD) of virulent *Y. pestis* CO92 at 28 days after the second immunization. Survival was monitored for 2 weeks after bubonic challenge. B. Mice were intranasally (i.n.) challenged with 4.1×10^3 CFU (LD) or with 4.4×10^4 CFU (HD) of virulent *Y. pestis* CO92 at 28 days after the second immunization. Survival was monitored for 2 weeks after pneumonic challenge.

Figure 4.

Humoral immune responses to PsaA and SOMPs in immunized mice surviving the post-intranasal challenge with *Y. pestis*. The humoral immune responses were measured by ELISA. Mice were orally immunized with *Salmonella* strain χ 9558(pYA3705) (PsaA) and χ 9558(pYA3342) (control) on days 0 and 9 and intranasally (i.n.) challenged with LD or HD of *Y. pestis* at 28 days after the second immunization. A. Serum IgG total response to PsaA. B. Subclasses (IgG1 and IgG2a) of anti-PsaA serum. C. *S. Typhimurium* anti-SOMP IgG. The data represent the endpoint of antibodies in pooled pre-immune serum (PI) and in immunized mice that survived i.n. challenge with virulent *Y. pestis*. Error bars represent variations between triplicate wells. The statistical significance was calculated by one-way ANOVA and Tukey's post-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).