Published in final edited form as:

Vaccine. 2016 May 05; 34(21): 2410–2416. doi:10.1016/j.vaccine.2016.03.094.

Multiple antigens of *Yersinia pestis* delivered by live recombinant attenuated *Salmonella* vaccine strains elicit protective immunity against plague

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Abstract

Based on our improved **novel** *Salmonella* vaccine delivery platform, we optimized the recombinant attenuated *Salmonella* Typhimurium vaccine (RASV) χ 12094 to deliver multiple *Y. pestis* antigens. These included LcrV196 (amino acids, 131 to 326), Psn encoded on pYA5383 and F1 encoded in the chromosome, **their** synthesis did not cause adverse effects on bacterial growth. Oral immunization with χ 12094(pYA5383) simultaneously stimulated high antibody titers to LcrV, Psn and F1 in mice and presented complete protection against both subcutaneous (s.c.) and intranasal (i.n.) challenges with high lethal doses of *Y. pestis* CO92. Moreover, no deaths or other disease symptoms were observed in SCID mice orally immunized with χ 12094(pYA5383) over a 60 day period. Therefore, the trivalent *S. Typhimurium*-based live vaccine shows promise for a next-generation plague vaccine.

Keywords

Trivalent vaccine; recombinant *Salmonella* vaccine; plague; LcrV; F1 and Psn

1. Introduction

Plague is a **notorious** disease which has claimed over 200 million human lives in three great pandemics [1]. Despite improved living standards and health services, plague still remains endemic in a substantial number of countries [2]. *Yersinia pestis* – a Gram-negative, non-motile, nonsporulating, bipolar staining coccobacillus – is the etiologic agent of plague [3]. Plague is enzootic in nature, primarily circulated by infected rodents and their fleas, which can incidentally infect humans [2]. This route of indirect transmission through the bite of a flea results in bubonic plague, which can cause high mortality if left untreated [4, 5]. Pneumonic plague is due to spread from infection of an initial bubonic plague to lung or

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inhalation of droplets containing infective *Y. pestis*, and can be transmitted from human-to-human through aerosols. Untreated pneumonic plague has a mortality rate from 90–100% [6].

In addition to natural infections, use of *Y. pestis* as a biological weapon has occurred in the past and this bacterium is considered to be among the top five bioweapons [7]. On account of the short incubation period of pneumonic plague and its ability to progress rapidly to a fatal infection, victims can become the source of secondary infections as indicated by historical plague epidemics [1]. Although treatment with antibiotics is effective for post-exposure prevention of disease, *Y. pestis* strains resistant to eight antibiotics have been isolated from plague patients in Madagascar [8]. Moreover, isolates obtained from **Mongolia** in a recent study further corroborate the existence of naturally occurring, multi-drug resistant variants of *Y. pestis* [9]. Therefore, there is an urgent need for effective means of pre-exposure and post-exposure prophylaxis.

During the past ten years, our group has endeavored to develop a vastly improved array of means to enhance the safety, efficacy and utility of *Salmonella* antigen delivery vaccines [10, 11]. We found that recombinant attenuated *Salmonella* vaccine (RASV) strains synthesizing LcrV196 or Psn individually protected mice against subcutaneous (s.c.) or intranasal (i.n.) challenge with *Y. pestis* CO92 [12]. Also, immunization with a RASV delivering YadC induced partial protection in mice against bubonic plague, but not pneumonic plague [13]. However, YadC synthesis is toxic to bacteria, and can seriously hinder bacterial growth. Moreover, significant protection could not be shown with a YadC and LcrV196 combination when delivered by RASV strains (unpublished data). Although it is doubtful that F1 antigen encoded by *cafI* is a necessary virulence antigen **due to the fact that anti-F1 negative strains of *Y. pestis* cause significant disease in animal models of infection** [14–17], vaccination of mice with F1 (encoded by *cafI*) yields serum that agglutinates plague bacilli and protects mice and rats from s.c. challenge with *Y. pestis*. **Anti-F1 antibodies also protect** macaques against pneumonic plague by passive transfer of sera collected from F1-vaccinated rabbits [18]. Thus, including F1 in a multiple antigen combination should augment protective immunity against *Y. pestis* strains that display F1.

In this particular study, we used RASV strains to deliver multiple *Yersinia* antigens (LcrV196, Psn and F1) and assessed their ability to protect mice against challenge with a virulent plague strain.

2. Materials and Methods

2.1 Bacterial strains and growth media

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. Typhimurium* UK-1 strains were routinely cultured at 37°C in LB broth [19] or on LB agar. *S. Typhimurium* UK-1 mutant strains were supplemented with 50 µg/ml of Diaminopimelic acid (DAP), 0.05% arabinose or 0.1% mannose when necessary for bacterial growth as described in our previous work [12]. Carbohydrate-free nutrient broth (NB) was used for growth when determining LPS profiles. LB agar with 5.0% sucrose (Sigma-Aldrich) was employed for *sacB* based counter-selection. MacConkey plates with 1% lactose were used to

indicate sugar fermentation. For animal experiments, *S. Typhimurium* strains were cultured in LB broth with appropriate supplements. Overnight cultures were diluted 1:100 and grown with aeration (200 rpm) to an optical density at 600 nm of ~0.85. Bacteria were then centrifuged at $5,000 \times g$ for 15 min at room temperature and resuspended in buffered saline with 0.01% gelatin (BSG) [20]. LB agar plates were used to enumerate *S. Typhimurium* recovered from mouse tissues. *Y. pestis* strain CO92 was routinely grown in heart infusion broth (HIB) containing 0.2% xylose at 28°C for subcutaneous (s.c.) route of challenge, and at 37°C for intranasal (i.n.) challenge studies. All media were purchased from BD Difco (Franklin Lakes, NJ) unless otherwise indicated.

2.2. Plasmid constructions

The gene fragments, P_{lpp-lacO-2-psn} (P_{lpp-lacO-2} containing 6 extra bases between P_{lpp-lacO} and the start codon of *psn*) and P_{lrc-bla ss-lcrV196-bla CT} transcribed in the same direction were cloned into plasmid pYA3620 to form pYA5383 (Figure 1A). The DNA sequences of all DNA inserts were confirmed by DNA sequence analysis. All primers were listed in Table S1 of Supplementary Materials and Methods.

2.3. Construction of bacterial mutant strains

S. Typhimurium mutant strains were constructed using conjugation with suicide vectors [13]. Parental *S. Typhimurium* strains were mated on LB agar plates containing DAP (50 µg/ml) with the *E. coli* host strain χ 7213 harboring the relevant suicide vector. Transconjugants were selected by growth on LB agar containing antibiotics to which the suicide vector conferred resistance without DAP. Conjugants with the *asdA* mutation were streaked onto antibiotic-containing selenite cysteine agar plates supplemented with DAP to inhibit *E. coli* growth. Defined deletion mutations with and without insertions were confirmed by PCR and phenotypic verification. Table 1 lists the constructed strains χ 12042 and χ 12094.

2.4. Determination of plasmid stability

Plasmid stability in strains χ 12042(pYA5383) and χ 12094(pYA5383) were determined with LB broth supplemented with 0.05% arabinose, 0.1% mannose under selective (without DAP) vs nonselective conditions (with DAP). Vaccine strains grown overnight (T₀) were diluted 1:1000 into pre-warmed LB supplemented as described above and grown with aeration for 12 h at 37°C. This process was repeated for approximately 50 generations (last subculture is called T₅) and the proportions of cells retaining the Asd⁺ plasmids were determined for each culture. The cultured cells from each successive culture were diluted, spread onto LB agar plates supplemented with 0.05% arabinose, 0.1% mannose and 50 µg/ml DAP. 100 colonies from each culture from each time were picked and patched onto LB agar supplemented with 0.05% arabinose, and 0.1% mannose, with or without 50 µg/ml DAP. Percentage of clones retaining the plasmids from each culture was determined by counting the colonies grown on LB agar with and without DAP. Continued ability of these clones to synthesize Lcrv-196, Psn and/or F1 was also checked after 50 generations.

2.5. Growth kinetics

The growth rates of strains χ 12042(pYA5383), χ 12094(pYA5383), χ 12042(pYA3841) and χ 12042(pYA3620) were determined by growing the strains in LB supplemented with 0.05% arabinose and 0.1% mannose. Briefly, overnight cultures were diluted 1:1000 or 1:10000 into 250 μ l of pre-warmed LB media supplemented as above and grown at 37°C using Bioscreen C MBR (Oy Growth Curves Ab Ltd., Finland). 250 μ l culture volumes were added in triplicates to honeycomb multi-well plates. The Bioscreen reader was set to measure optical density (OD) of the cultures at 30 min intervals for 24 h at OD₆₀₀ nm and incubation temperature of 37°C.

2.6. Protein synthesis and purification

E. coli TOP10 carrying pYA4836 (*lcrV*-6xHis), pYA5049 (*psn*-6xHis) and pYA5338 (*cafI*-6xHis) was grown overnight at 37°C in LB broth supplemented with 100 μ g/ml ampicillin. The procedures for protein synthesis and purification have been described in our previous study [21].

2.7. SDS-PAGE and western blot analysis

S. Typhimurium strains, χ 12042(pYA5383), or χ 12094(pYA5383), or empty plasmids were cultured in LB containing 0.05% arabinose and 0.1% mannose at 37°C. When the bacteria reached an OD₆₀₀ of 0.8, 1 mM IPTG was added to the cultures to induce heterologous *Yersinia* protein syntheses. Protein samples from bacterial pellets and culture supernatants were separated and analyzed by SDS-polyacrylamide gel electrophoresis with transfer to nitrocellulose membranes as previously described [12]. Proteins were detected by rabbit polyclonal anti-LcrV antibodies, rabbit polyclonal anti-Psn antibodies and mouse monoclonal anti-F1 antibodies (Genway).

2.8. Immunization of mice

All animal experiments were approved by the Arizona State University Institutional Animal Care and Use Committee. Female BALB/c mice (**n=10/group**), **aged 7 weeks**, were purchased from Charles River Laboratories (Wilmington, MA). Immunization procedures followed the previous description [12]. Briefly, mice were deprived of food and water for 4 h prior to the immunization and re-supplied 30 min later. Mice were orally immunized with 20 μ l BSG containing 10⁹ CFU of each strain, or with 20 μ l BSG as the negative control on day 0 and boosted on day 10. Blood samples were collected individually on days 0, 14 and 28, and serum was collected individually after centrifugation for **individual** analysis of antibody responses. Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc, Rochester, NY, USA) were coated with either purified recombinant LcrV (100 ng/well), Psn (100 ng/well) or F1 (100 ng/well) and incubated overnight at 4°C. The procedures for measuring antibody titer were followed as described in our previous reports [12, 22].

2.9. Challenge with *Y. pestis*

The s.c. and i.n. LD50s of *Y. pestis* CO92 in BALB/c mice were <10 CFU and ~100 CFU, respectively [12]. Challenge was performed 4 weeks after the second immunization (day 38). For s.c. challenge, each animal received a dose of 5.7×10^3 CFU of *Y. pestis* CO92 (~

570 LD₅₀) in 100 µl BSG, freshly grown at 28°C in HIB containing 0.2 % xylose. For i.n. challenge, mice were anesthetized with a cocktail of ketamine/xylazine. Then, each mouse received an intranasal dose of $\sim 5 \times 10^3$ CFU of *Y. pestis* CO92 (50 LD₅₀) in 20 µl BSG, freshly grown at 37°C in HIB containing 0.2% xylose and 2 mM CaCl₂. Mice were observed daily, and mortality was recorded for 15 days after the challenge.

2. 10. Test with SCID mice

RASV strains χ 12042 and χ 12094 were evaluated for safety using 7 week old SCID Beige mice (n=6 or 8/group). Mice were orally administered with a single dose of 1×10^9 CFU of strain χ 12042 harboring one of the three plasmid, pYA3841, pYA3620, pYA5383 or strain χ 12094(pYA5383). These mice were observed for signs of mortality and morbidity for 60 days.

2.11. Statistical analysis

Antibody responses were compared using two-way ANOVA. The log-rank (Mantel-Cox) test was used for analysis of the survival curves. Data are expressed as means $\pm$ SD. P-value <0.05 was considered significant.

3. Results

3.1. Construction of improved RASVs to deliver multiple protective antigens of *Y. pestis*

While conducting human trials using three *S. Typhi* RASV strains, findings from our studies led us to recognize the crucial role of certain mutations in immunogenicity and safety of RASV strains [23]. One of the most significant changes was the substitution of *relA198::araC*P_{BAD} *lacITT* mutation by *relA197::araC*P_{BAD} *lacITT* mutation to increase the level of antigen synthesis in vivo, which is associated with a lower level of LacI synthesis during growth in the presence of arabinose [24]. The 20 gene (*wza-wcaM*)-8 deletion precludes synthesis of colanic acid (as does the (*gmd-fcl*)-26) but also the O-antigen capsule to further reduce biofilm formation. Furthermore, the (*wza-wcaM*)-8 mutation increases the level of protective antigen synthesis and thus, enhances immune responses [25]. The P_{fur81::TT} *araC*P_{BAD} *fur* mutation was replaced by the P_{fur33::TT} *araC*P_{BAD} *fur* mutation since strains with the latter mutation colonize better and induce higher immune responses [26]. Notably, our recent findings indicate that RASV strains with *pmi*, P_{fur33::TT} *araC*P_{BAD} *fur*, and P_{crp527::TT} *araC*P_{BAD} *crp* are safe in immunocompromised mice (unpublished data). RASV *asdA* mutants are DAP dependent, and require a plasmid with the wild-type *asdA* gene to establish complementation [10, 27, 28]. Here, we have constructed *S. Typhimurium* strains using conjugation with suicide vectors containing these optimal mutations to form χ 12042 (Table 1).

We found that the plasmid containing P_{lpp-lacO}.*cafIM-cafIA-cafI* or even P_{lpp-lacO-2}.*cafIMcafIA-cafI* (P_{lpp-lacO-2} containing 6 extra bases before the start codon of *cafIM* for reducing F1 synthesis) in χ 12042 resulted in F1 synthesis at high levels, which adversely affected bacterial growth and induced poor protective immunity (unpublished data). Thus, we integrated the P_{lpp-lacO}.*cafIM-cafIA-cafI* construction into the χ 12042

chromosome to obtain χ 12094 (Table 1) to reduce the level of F1 synthesis. Results indicate that F1 was detected but did not decrease the growth of bacteria.

3.2. RASV strains with a single *Asd*⁺ plasmid synthesizing multiple antigens (LcrV196 and Psn)

Synthesis of Psn in a high copy number plasmid (pBR *ori*) is somewhat toxic. Usually we express *psn* in a low copy plasmid (p15A *ori*), pYA3996 [12]. Plasmid pYA3841, a pBR *ori* *Asd*⁺ plasmid, encodes the *bla*-SS-*lcrV196*-CT fusion, in which the N-terminal end of the *lcrV196* sequence is fused with the N-terminal secretion signal sequences (SS) of β -lactamase and the C-terminus of LcrV196 is fused with C-terminal 22 amino acids of β -lactamase [29]. Immunization with RASV strains harboring pYA3841 induced **statistically significant protection** against virulent *Y. pestis* challenge [29]. In order to deliver multiple antigens, we constructed a pBR *ori* plasmid pYA5383 containing P_{lpp-lacO-2}-*psn* (P_{lpp-lacO-2} containing 6 extra bases before the start codon of *psn* for reducing Psn synthesis) and P_{trc}-*bla ss-lcrV196-bla CT* transcribed in the same direction (Fig. 1A). Upon introduction of pYA5383 into χ 12042, LcrV196 and Psn were both optimally produced, and did not affect the growth of the bacteria (Fig. 1B and D). LcrV196, Psn and F1 synthesized in χ 12094(pYA5383) also did not show adverse effects on bacterial growth (Fig. 1C and D). Moreover, growth of RASV strains harboring different plasmids was not dependent on initial dilution rates (1:1000 or 1:10000) (Fig. D and Fig. S1A).

3.3. Synthesis and stability of LcrV196, Psn and F1

The expression profile of χ 12042 and χ 12094 expressing *lcrV196* and *psn* from pYA5383 was checked by growing the strains in LB broth supplemented with 0.05% arabinose and 0.1% mannose. χ 12042 carrying the empty vector did not produce any protein that reacted with anti-LcrV, anti-Psn or anti-F1 antibodies. A protein band with approximate molecular mass of 25 kDa in total cell extracts reacted with the anti-LcrV antibody. Psn (with a molecular mass of 73.7 kDa) and F1 (17 kDa) were also detected in whole-cell lysates by immunoblotting with rabbit anti-Psn serum or mouse anti-F1 serum. Also, the synthesis of all three proteins was stably maintained for 50 or more generations when the strain was grown in LB broth as described in Materials and Methods (Fig. S1B, C and D).

3.4. Serum antibody responses to recombinant antigens

Upon oral vaccination with RASV strains delivering recombinant antigens, mice in all groups, except the χ 12042(pYA3620), developed significant IgG serum antibodies to each antigen, which peaked around 28 days after immunization. Immunization with χ 12042(pYA3841), χ 12042(pYA5383) and χ 12094(pYA5383) exhibited significantly higher IgG titers against rLcrV than did χ 12042(pYA3620) at both week 2 and week 4 post immunization (Fig. 2A). Immunization with χ 12042(pYA5383) and χ 12094(pYA5383) primed significantly higher anti-Psn IgG titers than did χ 12042(pYA3620) (Fig. 2B). Only immunization with χ 12094(pYA5383) produced significant high anti-F1 IgG titers as well (Fig. 2C). High antibody subtype titers (IgG1 and IgG2a) were found to be developed by all antigen-expressing groups (Fig. S2). However, there was no significant difference between IgG1 and IgG2a subtypes with regard to any of the three recombinant antigens, although the

corresponding titers were significantly higher than those of the control strain χ 12042(pYA3620) (Fig. S2).

3.5. Protection against *Y. pestis* challenge

Mice were orally vaccinated with different RASV strains and then challenged with a lethal dose of *Y. pestis* CO92 by s.c. or i.n route according to procedures described in Materials and methods. Immunization with χ 12094(pYA5383) delivering *Yersinia* antigens LcrV-196, Psn and F1 encoded in the chromosome of the χ 12094 provided complete protection for mice against ~ 570 LD₅₀ of *Y. pestis* CO92 by s.c. challenge, whereas immunization with χ 12042(pYA5383) delivering LcrV-196 and Psn or χ 12042(pYA3841) only delivering LcrV-196 as a positive control provided 50% or 70% protection against the same doses by s.c. challenge, respectively (Fig. 3A). For pneumonic protection, χ 12094(pYA5383) or χ 12042(pYA5383) immunization afforded 60% or 40% protection for mice against 50 LD₅₀ of *Y. pestis* CO92 by i.n. challenge, which was significantly higher than that of χ 12042(pYA3841) immunization (Fig. 3B). All mice inoculated with either BSG or control strain χ 12042(pYA3620) as a negative control succumbed to the challenge both via s.c. and i.n. routes (Fig. 3A and B).

3.6. Safety assessment of RASV strains

The safety profile of the vaccine strains χ 12042(pYA5383) and χ 12094(pYA5383) was assessed using SCID Beige mice following oral inoculation. Mice (n=6) that received 0.65×10^9 CFU of control strain χ 12042(pYA3620) expressing no recombinant antigens showed 100% survival. Similarly, inoculation with 1.19×10^9 CFU of χ 12094(pYA5383) group (n=8) also exhibited zero percent mortality (Fig. 4). However, 75% of mice (n=8) administered with 1.17×10^9 CFU of χ 12042(pYA5383) survived by the end of 60 day observation period and 50% of the mice (n=8) administered with 1.1×10^9 CFU of χ 12042(pYA3841) died over a period of 50 days post inoculation (Fig. 4). Survival of χ 12042(pYA5383) or χ 12042(pYA5383) was lower than χ 12042(pYA3620) and χ 12094(pYA5383), but there were no significant differences in statistical analysis (Fig. 4). The above data looks somewhat promising since χ 12094(pYA5383) was found to be safe in immunodeficient mice. However, survival results obtained using plasmids pYA5383 and pYA3841 in χ 12042 necessitate further attenuation of these strains, in order to be usable for immunocompromised subjects in future.

4. Discussion

Subunit vaccines based on rF1 and rLcrV antigens have progressed through Phase I and II clinical trials, but the vaccines failed to protect African green monkeys against pneumonic plague, which poses questions about their ability to protect humans [30–32]. However, F1-negative *Y. pestis* strains isolated from natural sources [33] caused experimental fatal disease in mouse [34] and African green monkeys inhaling these F1-negative and/or F1-positive strains of *Y. pestis* died 4 to 10 days post exposure consistent with primary pneumonic plague [35]. ***Y. pestis* is easily genetically manipulated** to create strains that lack highly immunogenic traits (F1[−] *Y. pestis*). The *cafI* *Y. pestis* was not only fully virulent in animal models of bubonic and pneumonic plague but also broke through immune responses

generated with live, attenuated strains of *Y. pestis* or F1 subunit vaccines [18, 36]. Additionally, antibodies raised against LcrV_{D27} were unable to block the type III injection of *Y. enterocolitica* strains, but fortunately did not allow *Y. pestis* expressing *lcrV*_{W22703} or *lcrV*_{WA-314} strains to escape LcrV-mediated plague protective immunity in the intravenous challenge model [37]. However, the *lcrV* polymorphisms that maintain the functional attributes of the type III secretion pathway may result in variants that escape plague protective immunity [38, 39]. Therefore, combination of F1 and LcrV only for a human vaccine may not sufficiently guarantee long-term defense against plague in humans [12, 14, 30–32, 35, 39].

Similarly, only LcrV and F1 presented by *Salmonella* may be insufficient to combat weaponized or naturally occurring *Y. pestis*, leading us to evaluate additional antigens. In addition to LcrV and F1, six other *Y. pestis* antigens individually, namely, Psn [12], HmuR [12], PsaA (pH 6 antigen) [40], YadC [41], Pla, and YopE (unpublished data) have been vectored by *Salmonella* in our research studies. However, only immunization with RASVs delivering Psn [12] or YadC afforded partial protection against *Y. pestis* challenge. Then, a pool of LcrV, F1, Psn and YadC showed the synergistic effects of multiple antigens (LcrV, F1 and Psn) in enhancing the protective immunity against both i.n. and s.c. *Y. pestis* CO92 challenge, when delivered using RASV strains (Fig. 3), but not YadC combination with LcrV (unpublished data). One reason described above was toxicity of YadC, and another may be too many antigens synthesized and delivered by one RASV cell, hinders growth of the RASV strain and further results in reduction of its immunogenicity. Recent work in our group showed that immunization with a mixture of the two RASVs, one delivering a carboxyl-terminal fragment of alpha toxin of *Clostridium perfringens* and the other a GST-NetB fusion protein induced protective immunity against *C. perfringens* in broiler chicks [42]. A similar approach could be applied for a mixture of two RASVs, one delivering LcrV, F1 and Psn antigens and the other another *Y. pestis* protective antigen, such as YopD [43], YscF [44], Ail [45] or YadC [41] to induce an optimal protective immune response.

Immunization with χ 12094(pYA5383) delivering LcrV-196, Psn and F1 and χ 12042(pYA5383) delivering LcrV-196 and Psn, produced similar amount of anti-LcrV antibody as immunization with the control strain χ 12042(pYA3841) delivering single LcrV196 in mice (Fig. 2), but immunization with χ 12094(pYA5383) afforded significantly higher protection against both s.c. and i.n. challenge with lethal doses of *Y. pestis* CO92 than did χ 12042(pYA3841), which suggested that delivering Psn and F1 with LcrV synergistically enhanced protective immunity against plague. Fetherston, et al. showed that LD_{50s} for *psn Y. pestis* strain in mouse models of bubonic and pneumonic plague were $>2.6 \times 10^7$ CFU (1×10^6 fold more than the LD₅₀ for wild-type *Y. pestis*) and 1.1×10^4 CFU (100 fold times the LD₅₀ for wild-type *Y. pestis*), respectively [46]. Whereas, immunization with χ 12042(pYA5383) provided less protection against s.c. challenge with a lethal dose of *Y. pestis* CO92, but better protection against i.n. challenge with a lethal dose of *Y. pestis* CO92. Current understanding is not clear. Moreover, χ 12094(pYA5383) was found to be considerably safe in immunocompromised mice compared to χ 12042(pYA5383) and χ 12042(pYA3841) (Fig. 4), although there was no statistic difference in safety evaluation. Based on the studies of *S. Typhimurium* vaccine strains, we will imprint the

same perfected genotypes into *S. Paratyphi A* as vaccines for delivering multiple protective antigens for a Phase I clinical trial.

To sum up, a trivalent *S. Typhimurium*-based live vaccine confers significantly high protection against bubonic and pneumonic plague in mice and shows promise for next-generation plague vaccines. Additionally, strategies developed herein offer the potential to use RASVs as vaccine platforms for construction of multivalent vaccines effective against a variety of human pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Stephen Forbes for SCID mice breeding. This work was supported by National Institutes of Health grants R01AI057885 and R01AI093348 to R.C. from the National Institute of Allergy and Infectious Diseases.

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Fig. 1. Recombinant *S. Typhimurium* vaccine strains deliver multiple *Y. pestis* antigens. (A). *Asd*⁺ vector pYA5383 expressing *lcrV196* (encoding truncated LcrV, aa 131 to 326) and *psn*. (B). Western blot showing presence of LcrV196 and Psn in different RASV strains, *χ12042(pYA3620)*, *χ12042(pYA5383)* and *χ12094(pYA5383)*. M, molecular-mass markers are labeled. (C) Western blot showing presence of F1 in different RASV strains, *χ12042(pYA5383)* and *χ12094(pYA5383)*. The molecular mass of the F1 monomer, 17 kDa. (D). Determination of growth curves of RASV strains harboring different plasmids. 3 ml cultures of each strain grown overnight were diluted 1:1000 into 250 μl of prewarmed LB media supplemented with 0.05% arabinose and 0.1% mannose and grown at 37°C using Bioscreen C MBR. 250 μl culture volumes were added in triplicates to honeycomb multi-well plates. The Bioscreen reader was set to measure optical density of the cultures at 30-min intervals for 24 h at OD₆₀₀ nm and incubation temperature of 37°C.

Fig. 2.

Antibody responses to recombinant LcrV, Psn and F1 determined by ELISA. The data represent IgG antibody levels induced in groups of 10 mice orally immunized with BSG, $\chi 12042(pYA3620)$, $\chi 12042(pYA3841)$, $\chi 12042(pYA5383)$ or $\chi 12094(pYA5383)$ at the indicated weeks after immunization. (A) Total IgG responses to LcrV. (B) Total IgG responses to Psn. (C) Total IgG responses to F1. Error bars represent standard deviations. ***, $P < 0.0001$. The sera from 10 mice were individually analyzed.

Fig. 3.

Protective efficacy in BALB/c mice orally immunized with RASV strains synthesizing LcrV, Psn or F1 antigens against *Y. pestis* challenge. Groups of 10 BALB/c mice were orally immunized with 1×10^9 CFU of χ 12042(pYA3620) without delivering antigen as a negative control, χ 12042(pYA3841) delivering LcrV196 as a positive control, χ 12042(pYA5383) delivering LcrV196 and Psn or χ 12094(pYA5383) delivering LcrV196 and Psn encoded on the plasmid and F1 encoded in the chromosome of χ 12094. Mice were challenged 4 weeks after the second immunization. Mortality was monitored for 15 days after challenge. (A) Mice were challenged with 5700 CFU (~ 570 LD₅₀) of virulent *Y. pestis* CO92 by s.c. administration. The χ 12094(pYA5383) vaccination group was significantly different from control groups including the pYA3620 group and BSG (****, $P < 0.0001$). χ 12094(pYA5383) vaccination group was significantly different from χ 12042(pYA5383) group (*, $P = 0.0136$). (B) Mice were challenged with 5000 CFU (~ 50 LD₅₀) of virulent *Y. pestis* CO92 by i.n. administration. The χ 12094(pYA5383) vaccination group was significantly different from control groups including the pYA3620 group and BSG (****, $P < 0.0001$). χ 12094(pYA5383) vaccination group was significantly different from χ 12042(pYA3841) group (*, $P = 0.0156$).

Fig. 4.

Safety assessment of RASV strains in SCID mice. A single dose of 10^9 CFU $\chi 12042(pYA3620)$, $\chi 12042(pYA3841)$, $\chi 12042(pYA5383)$ or $\chi 12094(pYA5383)$ were orally administrated to SCID mice (B, T and NK cells deficient). These mice were observed for signs of mortality and morbidity for 60 days. There was no significant difference among each group (Log-rank (Mantel-Cox) test).

Table 1

Strains and plasmids used in this study

Strain or Plasmid	Genotype or relevant characteristics	Derivation or source
Strains		
<i>E. coli</i>		
TOP10	F ⁻ <i>mcra</i> (<i>mir-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> M15 <i>lacX74 recA1 araD139 (ara-leu)-7697 galU galK rpsL (Str^r) endA1 nupG</i>	Invitrogen [47]
χ 6212	F ⁻ λ Φ 80 (<i>lacZYA-argF</i>) <i>endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 asdA4</i>	
<i>Salmonella</i>		
Typhimurium UK-1		
χ 12042	<i>pmi-2426 (wza-wcaM)-8 asdA33 relA197::araCP_{BAD} lacI^{TT} P_{fur33}::TT araCP_{BAD} fur P_{cp527}::TT araCP_{BAD} crp</i>	This study
χ 12094	<i>pmi-2426 (wza-wcaM)-8::P_{pp-lacO} cafI/M-cafI A-cafI asdA33 relA197::araCP_{BAD} lacI^{TT} P_{fur33}::TT araCP_{BAD} fur P_{cp527}::TT araCP_{BAD} crp</i>	This study
<i>Y. pestis</i>		
<i>Y. pestis</i> CO92	Pgm ⁺ , pMT1, pPCP1, pCD1Ap	[48]
Plasmids		
pBAD/hisB	Expressing vector	Lab collection
pYA3342	Asd ⁺ pBR <i>ori</i>	[47]
pYA3841	Asd ⁺ pBR <i>ori</i> , the <i>bla</i> -SS- <i>lcrV196</i> -CT gene fragment under P _{tec} promoter	[49]
pYA4836	The <i>lcrV</i> gene of <i>Y. pestis</i> KIM6+(pCD1Ap) fused with a C-terminal 6 $\times$ His cloned into the <i>NcoI</i> and <i>HindIII</i> sites of plasmid pBAD/HisB	[39]
pYA5049	The <i>psn</i> gene of <i>Y. pestis</i> KIM6+(pCD1Ap) cloned into the <i>NcoI</i> and <i>HindIII</i> sites of plasmid pBAD/HisB	This study
pYA5333	The P _{tec} promoter of pYA3342 was replaced by P _{pp-lacO-2} (containing 6 extra bases in the C-terminal end of P _{pp-lacO})	This study
pYA5338	The <i>cafI/M-cafI A-cafI</i> -6 $\times$ His was cloned under control by the P _{tec} promoter of pYA3342	This study
pYA5381	The native <i>psn</i> was cloned into the <i>NcoI</i> and <i>HindIII</i> sites of plasmid pYA5333	This study
pYA5383	The P _{tec} - <i>bla</i> -SS- <i>lcrV196</i> -CT amplified from pYA3841 was cloned into the <i>SacI</i> and <i>XmaI</i> sites of pYA5381	This study