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Evaluation of Psn, HmuR and a modified LcrV protein delivered to mice by live attenuated *Salmonella* as a vaccine against bubonic and pneumonic *Yersinia pestis* challenge

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Abstract

We evaluated the ability of *Yersinia pestis* antigens HmuR, Psn and modified forms of LcrV delivered by live attenuated *Salmonella* strains to stimulate a protective immune response against subcutaneous or intranasal challenge with *Y. pestis* CO92. LcrV196 is a previously described truncated protein that includes aa 131–326 of LcrV and LcrV5214 has been modified to replace five key amino acids required for interaction with the TLR2 receptor. Psn is the outer membrane receptor for the siderophore, yersiniabactin, and the bacteriocin, pesticin. Mice immunized with *Salmonella* synthesizing Psn, LcrV196 or LcrV5214 developed serum IgG responses to the respective *Yersinia* antigen and were protected against pneumonic challenge with *Y. pestis*. Immunization with *Salmonella* synthesizing Psn or LcrV196 was sufficient to afford nearly full protection against bubonic challenge, while immunization with the strain synthesizing LcrV5214 was not protective. Immunization with *Salmonella* synthesizing HmuR, an outer membrane protein involved in heme acquisition in *Y. pestis*, was poorly immunogenic and did not elicit a protective response against either challenge route. These findings indicate that both Psn and LcrV196 delivered by *Salmonella* provide protection against both bubonic and pneumonic plague.

1. Introduction

The genus *Yersinia* contains three species pathogenic for humans: *Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* [1,2]. *Y. pestis* is the causative agent of plague. The most common way that humans get infected is via bites from an infected flea, leading to bubonic plague. Bubonic plague can develop into the more serious and highly transmissible pneumonic form of plague. Individuals with pneumonic plague can transfer the disease through aerosols, leading to rapid spread of the disease.

Y. pestis harbors the 70-kb low calcium response plasmid that encodes essential virulence factors including the type III secretion system [3]. LcrV is a secreted, multifunctional protein that is central to the activity of the type III secretion system apparatus. LcrV affects

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effector secretion by binding the negative regulator LcrG and, in conjunction with YopB and YopD, is essential for the translocation of effectors into eukaryotic cells [4]. In addition, LcrV may contribute to pathogenicity via TLR2-mediated IL-10 induction [5]: LcrV causes TNF- α suppression in macrophages in a CD14 and TLR2-dependant manner by inducing IL-10 [6–9]. Other studies indicate that TLR6 is essential for IL-10 induction [10].

LcrV is highly immunogenic, inducing a protective immune response, and is therefore a component of most plague vaccines. It is highly conserved among the *Yersinia*, although some protein polymorphism has been described in *Y. pseudotuberculosis* and *Y. enterocolitica* strains [11–13]. Although immunization with purified LcrV can induce protective immunity, the associated suppression of host defense mechanisms through CD14/TLR2/TLR6 receptors may limit the value of LcrV as a human vaccine. To overcome this potential shortcoming, we designed a truncated version of LcrV, LcrV196 and a mutated version of LcrV, LcrV5214; both of these constructs were designed to eliminate the TLR2 binding receptor, either by deletion or modification of the relevant amino acids.

Psn is a TonB-dependent outer membrane protein that is chromosomally encoded by *psn* (called *fyuA* in *Y. enterocolitica*) in the high pathogenicity island [14,15]. This pathogenicity island is present in highly pathogenic strains of *Yersinia* such as *Y. enterocolitica* 1B, *Y. pseudotuberculosis* and *Y. pestis* [14,16]. Psn is the outer membrane receptor for yersiniabactin, a siderophore that is essential for in vivo growth, and *psn* expression, responsive to iron availability, is modulated by the Fur repressor [17]. Strains which lack *psn* are avirulent when administered by the subcutaneous route [18,19] and are highly attenuated when administered intranasally [19].

HmuR is a TonB-dependent outer-membrane protein that is also regulated by Fur [15,20]. HmuR is required for the utilization of hemin and mammalian heme-protein complexes as a source of iron [21]. However, deletion of the *hmu* locus has no effect on virulence when mice are infected subcutaneously, intravenously or intranasally [22,23].

Live attenuated *Salmonella* strains were first developed as vaccines to prevent disease caused by *Salmonella* infections of both humans and animals [24]. Subsequently, genetically modified attenuated *Salmonella* strains were constructed for delivery of heterologous antigens. Orally administered *Salmonella* vaccines offer a variety of advantages over traditional vaccines, including stimulation of a strong mucosal response, important for protection against pathogens that colonize and/or enter the body through mucosal surfaces, stimulation of a systemic response, needle-free delivery and a relatively low cost of production [25]. Ongoing research in our laboratory has been directed toward improving the immunogenicity and stability of *Salmonella* delivery strains and methods of antigen expression. In this study we evaluated the protective efficacy of HmuR, Psn and modified LcrVs delivered by attenuated *Salmonella enterica* serovar Typhimurium. We compared the efficacy of two different recombinant attenuated *S. Typhimurium* vaccine (RASV) vector strains, χ 8501 and χ 9558. Strain χ 8501 is attenuated by a *crp* mutation. We have previously shown that immunization of mice with this strain expressing either *lcrV*196, a truncated form of *lcrV*, or *psn* can protect mice from bubonic challenge with virulent *Y. pestis* [26]. In this study, we extended these observations to evaluate the efficacy of these vaccine strains to protect against pneumonic challenge, evaluate a full-length *lcrV* with five amino acid changes designed to eliminate the interaction between LcrV and TLR2 and we examined the efficacy of *hmuR* expressed in χ 8501. In addition, we evaluated the efficacy of *lcrV* and *psn* expressed in strain χ 9558, a new generation vaccine delivery vector with a constellation of mutations designed to enhance both safety and immunogenicity [27–32].

2. Materials and methods

2.1 Bacterial Strains and growth media

Y. pestis strain CO92 [33] was used for challenge studies. *Yersinia* strains were routinely grown in heart infusion broth (Difco, Detroit, MI) at 28°C. All other strains used in this study are shown in Table 1. Bacteria were grown in LB broth [34], on LB agar or on MacConkey agar supplemented with 1% lactose. Diaminopimelic acid (DAP) was included in growth media at 100 µg/ml when necessary for growth of non-complemented *asd* strains and for plasmid stability tests. Arabinose (0.05%) and mannose (0.2%) were added for the growth of plasmid-harboring derivatives of strain χ 9558.

2.2 Construction of recombinant plasmids encoding *Y. pestis* genes

For the construction of pYA3995, a 2036 bp PCR fragment encoding *hmuR* was cloned into *Asd*⁺ plasmid pYA3342. The primers used for PCR were 5'-GGAATTCCGCTCCACTTCCGACCGTTTCC-3' and 5'-CGGATCCGCTACCACTGATAACTCACG-3'. These primers also encode an *EcoRI* or *BamHI* site (underlined), respectively. The *hmuR* gene was amplified using genomic DNA from *Y. pestis* KIM6+ [35] as the template, digested with *EcoRI* and *BamHI* and ligated into the *EcoRI* and *BamHI* sites of pYA3342.

To construct plasmid pYA4665, the complete *lcrV* coding sequence was PCR amplified from plasmid pJIT7 [36] and cloned into pUC19. Codons of *lcrV* were then optimized for expression in *Salmonella* using a combination of PCR mutagenesis and a QuickChange II site directed mutagenesis kit (Stratagene, La Jolla, CA). The modified codons were those encoding aa 184 (ATA to ATT), aa 233 (ATA to ATT), aa 236 (ATA to ATT), aa 241 (GGA to GGC), aa 246 (AGA to CGC), and aa 320 (CTA to CTC). The codon-optimized gene was cloned into *EcoRI*/*PstI* digested pYA3620 to generate plasmid pYA4661.

We further modified the *lcrV* coding sequence to target the amino acids involved in TLR2 and CD14 binding [6,12]. The QuickChange II site directed mutagenesis kit was used to modify codons in *lcrV* at positions 97 (E33Q) and 100 (E34Q) using the primer 5'-TCTTCAGTTTTACAACAATTGGTTCAGTTAGTC and its complement, at position 124 (K42Q) using the primer 5'-GTCAAAGATCAAAATATTGATATTTCC-3', and its complement, and at positions 610 (E204Q) and 613 (E205Q) using the primer 5'-GGTTATACAGATCAACAGATTTTAAAGCC-3' with its complement. The modified gene, *lcrV5214*, was digested with *EcoRI* and *PstI* and the resulting 998 bp fragment was ligated in *EcoRI*/*PstI*-digested pYA3620 to generate plasmid pYA4665.

The construction of plasmids pYA3841, encoding a *bla*-SS-*lcrV*196-CT fusion, and pYA3996, encoding *psn*, has been previously described [26].

The DNA sequence of all modified *Y. pestis* genes was confirmed by DNA sequence analysis. Nucleotide sequencing reactions were performed by the sequencing laboratory at Arizona State University using ABI Prism Fluorescent Big Dye terminators according to the instructions of the manufacturer (PE Biosystems, Norwalk, CT). Plasmids pYA3332, pYA3620, pYA3995, pYA3996, and pYA3841 were electroporated into *S. Typhimurium* strain χ 8501. The plasmids pYA3620, pYA3996, pYA3841 and pYA4665 were electroporated into *S. Typhimurium* strain χ 9558.

Plasmid stability tests in strains χ 8501 and χ 9558 were performed as previously described [37].

2.3 SDS PAGE and western blot analysis

To evaluate antigen expression, *S. Typhimurium* strains χ 8501 or χ 9558 expressing antigens or harboring control plasmids were cultured in LB broth at 37°C and harvested when the cultures reached an optical density at 600 nm (OD₆₀₀) of 0.8 by centrifugation at 10,000 g. The pellets were resuspended in Laemmli sample buffer containing 2 % 2-mercaptoethanol. Supernatants were passed through a 0.22 μ m filter and precipitated overnight with 20% trichloroacetic acid (TCA) (v/v) at 4°C. After centrifugation, the TCA pellet was resuspended in cold phosphate buffered saline (PBS), and acetone precipitated. The precipitates were washed with acetone, resuspended in 250 μ l PBS and stored at -20°C.

The proteins were separated by SDS-polyacrylamide gel electrophoresis as previously described [38] and transferred onto nitrocellulose sheets (Biorad, Hercules, CA) using a semi-dry system with Tris buffer (48 mM Tris, pH 9.2, 39 mM glycine, 1.3 mM SDS, 20% methanol). After overnight blocking at 4°C with 3% BSA in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20), recombinant proteins were selectively identified by western blot using rabbit anti-LcrV (1:5000) anti-Psn (1:5000), or anti-HmuR serum (1:1000) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). Antibody complexes were detected with the NBT-BCIP liquid substrate (Amresco).

2.4 Immunization of mice

All animal experiments were approved by the Arizona State University animal care and use committee. Female Swiss Webster (immunization with the strain χ 8501 expressing *Y. pestis* antigens) or BALB/c mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). Mice were deprived of food and water for 4 h prior to immunization and re-supplied 30 min after. *S. Typhimurium* strains were grown in LB broth with appropriate supplements at 37°C to an OD₆₀₀ of 0.9 and concentrated to 5×10^{10} CFU/ml in buffered saline containing 0.01% gelatin (BSG) [39]. Groups of mice were orally immunized with 20 μ l of RASV suspensions on days 0 and 10. Blood samples were collected on days 0, 21, 32, and at the end of the experiment. Eight animals received three subcutaneous injections with 20 μ g of rLcrV with incomplete Freund's adjuvant in 200 μ l at two-week intervals.

2.5 Colonization of mouse tissues

BALB/c mice were immunized with a single dose of strains χ 8501(pYA3620) (1.0×10^9 CFU), χ 8501(pYA3995) (1.0×10^9 CFU), χ 8501(pYA3996) (1.3×10^9 CFU), χ 8501(pYA3841) (2.5×10^9 CFU), χ 9558(pYA3620) (1.0×10^9 CFU), χ 9558(pYA3841), (1.2×10^9 CFU) or χ 9558(pYA4665) (1.7×10^9 CFU). Three mice from each group were euthanized by asphyxiation with CO₂ on days 3, 7, 10 and 15 post-immunization. The Peyer's patches, spleen and part of the liver were aseptically taken from each mouse, weighed and homogenized in PBS using a PowerGen 125 S1 homogenizer (Fischer Scientific, Pittsburgh, PA). Dilutions of these samples were spread onto LB and McConkey-1% lactose plates. The plates were incubated overnight at 37°C. Colonies were counted to determine the number of CFU recovered per gram of each organ.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc, Rochester, NY, USA) were coated with purified His₆-tagged LcrV (100 ng/well), His₆-tagged Psn (100 ng/well), His₆-tagged HmuR (100 ng/well), or *S. Typhimurium* LPS (100 ng/well) (Sigma) and incubated overnight at 4°C. The expression and purification of His₆-tagged LcrV from pHTV, His₆-tagged Psn from pPPH-2 and His₆-tagged HmuR has been previously described [20,40]. The plates were washed three times with PBS containing 0.1% Tween 20, blocked with PBS

containing 10% (v/v) SEA BLOCK Blocking buffer (Pierce) for 1 h at 37°C and then washed three times with PBS. The sera from all mice in a group were pooled, diluted in PBS and added to the plates. After 1 h incubation at 37°C, the plates were washed, followed by the addition of 100 µl of goat anti-mouse IgG(H+L), IgG₁, IgG_{2A} or IgA biotin conjugated (SouthernBiotech) (1:5000) and incubated 1 h at 37°C. The plates were washed again and streptavidin conjugated to alkaline phosphatase (SouthernBiotech) (1:4000), was added and the plates were incubated for 1 h at 37°C, washed five times, and the *p*-nitrophenyl phosphate chromogenic substrate (Sigma) for alkaline phosphatase was added. After 10 min, the reaction was stopped by the addition of 2M H₂SO₄. The optical density at 405 nm was measured using the automated ELISA plate reader (EL311SX, Biotech, Winooski, VT).

2.7 Challenge with *Y. pestis*

Challenge was performed 28 days after the second immunization (day 38). For subcutaneous challenge, each animal received a dose of approximately 60–6,000 CFU of *Y. pestis* CO92 freshly grown at 28°C in heart infusion broth (HIB) containing 0.2 % xylose. For intranasal challenge, mice were anesthetized with a cocktail of ketamine/xylazine, each mouse received a dose of 300–44,000 CFU of *Y. pestis* CO92 freshly grown at 37°C in HIB containing 0.2% xylose and 2 mM CaCl₂. Mice were observed daily, and mortality was recorded for 14 days after the challenge.

2.8 Statistical analysis

Statistical significance was determined by the χ^2 test, with $P < 0.05$ considered to be statistically significant.

3. Results

3.1 Synthesis of LcrV196, LcrV5214, Psn and HmuR

Expression of *lcrV196* in strain χ 8501 was described previously [41]. Expression of *lcrV196* and *lcrV5214* in RASV strain χ 9558 was evaluated by Coomassie brilliant blue-staining of SDS-PAGE gels and by western blot analysis (Fig. 1A, 1B). Strains carrying the empty vector plasmids did not produce any protein that reacted with the anti-LcrV antibody (Fig. 1B). Plasmids pYA3841 and pYA4665 encode fusions of *lcrV196* and *lcrV5214* to the DNA sequence encoding amino-terminal and carboxy-terminal secretion signals from β -lactamase that direct the protein products to the periplasm and supernatant. The supernatant fractions from χ 9558(pYA3841) and χ 9558(pYA4665) contained protein that reacted with the anti-LcrV antibody (Fig. 1B), indicating that the β -lactamase signal sequence was directing antigen secretion as expected. Supernatant fractions from strains carrying empty vector plasmids did not react with the anti-LcrV antibody (Fig. 1B). The amount of LcrV196 synthesized was similar in both χ 8501 and χ 9558 (Fig. 1B, lanes 1 and 2).

Synthesis of HmuR was evaluated in RASV strain χ 8501(pYA3995) (Fig. 1A, 1C). HmuR synthesized in χ 8501(pYA3995) was directed to the outer membrane (Fig. 1C, lane 3). Strains carrying the empty vector plasmid did not produce any protein that reacted with anti-HmuR antibody at the expected size (74.1 kDa).

Psn RASV strains χ 8501(pYA3996) and χ 9558(pYA3996) produced similar amounts of cell-associated Psn in both strains (Fig. 1A, Fig. 1D, lanes 1 and 5). The predicted size of Psn is 73.7 kDa. Psn is an outer membrane protein in *Y. pestis* [42]. We evaluated outer membrane fractions of χ 8501(pYA3996) and χ 9558(pYA3996) to determine whether Psn was also able to insert into the *Salmonella* membrane. Outer membrane fractions from both RASVs contained protein that reacted with the anti-Psn antibody (Fig. 1D, lanes 3 and 7), indicating that the secretion and membrane insertion signals in Psn can function in

Salmonella. Strains carrying the empty vector plasmids did not produce any protein in the 68–70 kDa range that reacted with anti-Psn antibody (Fig. 1D).

3.2 Colonization of mouse tissues

Groups of mice were immunized with strain χ 8501 synthesizing LcrV196 (pYA3841), HmuR (pYA3995) or Psn (pYA3996) as well as with RASV strain χ 9558 synthesizing LcrV196 (pYA3841), LcrV5214 (pYA4665) or Psn (pYA3996). At 3, 7 and 10 days after immunization, three mice from each group were euthanized, tissue samples taken and the number of bacteria per tissue was determined (Fig. 2). There was no significant difference in colonization within groups (χ 8501 or χ 9558 harboring different plasmids). However, plasmid-bearing χ 8501 strains achieved higher titers within each tissue than plasmid-bearing χ 9558 strains and the χ 9558 derivatives were cleared from all tissues more quickly than the χ 8501 strains.

3.3 Anti-LcrV, anti-Psn, anti-HmuR and anti-Salmonella LPS antibody responses in immunized mice

The serum IgG responses in mice immunized against the recombinant full length LcrV protein and Psn were determined by ELISA (Fig. 3A). No anti-LcrV or anti-Psn IgG was detected in mice receiving control strains χ 8501(pYA3342) and χ 9558(pYA3620). Mice orally immunized with strains expressing either *lcrV196* or *psn* developed high titers against LcrV and Psn, respectively. The anti-LcrV and anti-Psn serum titers increased between week 3 and week 5. Both *Salmonella* delivery strains expressing *lcrV196* elicited similar anti-LcrV titers at both time points. The titers for Psn induced by both strains were similar at 3 weeks, but greater at 5 weeks in mice immunized with χ 8501(pYA3996) than in mice immunized with χ 9558(pYA3996). Mice immunized with strain χ 9558 expressing either *lcrV196* or *lcrV5214* developed high titers against LcrV. The antibody responses to *Salmonella* LPS were also measured (Fig. 3B). After the second immunization, all immunized mice developed similar anti-LPS IgG antibody titers with no significant difference of magnitude between vaccine strains carrying an empty plasmid and those expressing antigens.

We evaluated the IgG isotypes IgG₁ (Fig. 3C) and IgG_{2a} (Fig. 3D) against LcrV and Psn. The levels of IgG_{2a} were greater than IgG₁ for all antigens at all time-points, indicating induction of a predominantly Th1 response.

We determined the IgG responses against HmuR in mice immunized with χ 8501(pYA3995) and χ 8501(pYA3342) (Fig. 3E). Mice immunized with χ 8501(pYA3995) developed a weak but detectable IgG response against HmuR. No anti-HmuR IgG was detected in mice receiving the control strain χ 8501(pYA3342). The anti-LPS IgG response in mice immunized with χ 8501(pYA3995) was similar to the responses observed in mice immunized with χ 8501(pYA3342) (Fig. 1B and data not shown).

3.4 Protection against *Y. pestis* challenge in mice immunized with χ 8501 derivatives

We immunized two groups of 16 mice each with strain χ 8501(pYA3620) (control), χ 8501(pYA3995) (*hmuR*), χ 8501(pYA3996) (*psn*) or χ 8501(pYA3841) (*lcrV196*) and boosted ten days later. Twenty-eight days after the boost, all mice were challenged with *Y. pestis* CO92. One group was challenged subcutaneously with either 60 CFU (8 mice) or 1300 CFU (8 mice) and the other group was challenged intranasally with either 443 CFU (8 mice) or 4450 CFU (8 mice). Mortality was recorded for 14 days after challenge. In preliminary experiments we determined that the subcutaneous and intranasal LD₅₀s of *Y. pestis* in BALB/c mice were <10 CFU and ~100 CFU, respectively (data not shown).

All mice immunized with strain χ 8501(pYA3841) (*lcrV196*) were protected against subcutaneous challenge at both doses, while 4 of 8 and 6 of 8 mice receiving χ 8501(pYA3996) (*psn*) survived subcutaneous challenge with low or high doses of *Y. pestis* (Table 2).

Immunization with strains χ 8501(pYA3995) (*hmuR*) and χ 8501(pYA3620) (control) provided the same low level of protection, which was not significantly different from the BSG control group ($P>0.05$). A similar level of protection against subcutaneous challenge with *Y. pestis* CO92 in mice immunized with χ 8501(pYA3620) was observed previously [26]. This result indicates that expression of *hmuR* in χ 8501 does not enhance the protection afforded by χ 8501 alone.

When mice were challenged intranasally, mice immunized with χ 8501 expressing *lcrV196* were protected (50% survival; $P<0.01$). Although three of eight mice immunized with χ 8501 expressing *psn* survived the low dose challenge, none of the mice survived the high dose challenge and the survival rate did not achieve statistical significance. None of the mice immunized with either χ 8501(pYA3620), χ 8501(pYA3995) or BSG survived challenge at either dose.

3.4 Protection against *Y. pestis* challenge in mice immunized with χ 9558 derivatives

To test our constructs delivered by strain χ 9558, we performed an experiment similar to the one described above. We immunized two groups of 16 mice per group with either χ 9558(pYA3620) (control), χ 9558(pYA3996) (*psn*), χ 9558 (pYA3841) (*lcrV196*) or χ 9558 (pYA4665) (*lcrV5214*) using the same immunization schedule, day 0 and a boost at day 10. In this experiment, we used an approximately 10-fold higher challenge dose (Table 3) for each route, 450 CFU or 5630 CFU for subcutaneous challenge and 4100 CFU or 44000 CFU for intranasal challenge. Mortality was recorded for 14 days after challenge. Based on the negative results obtained with χ 8501 expressing *hmuR*, we did not include that antigen in these experiments.

Mice orally immunized with RASV strains χ 9558(pYA3841) (*lcrV196*) or χ 9558(pYA3996) (*psn*) were significantly protected against subcutaneous challenge with *Y. pestis* CO92 ($P<0.01$). All positive control mice injected with purified LcrV survived the challenge as well. All mice orally inoculated with either BSG or control strain χ 9558(pYA3620) succumbed to the challenge. Unexpectedly, none of the mice orally immunized with χ 9558(pYA4665) (*lcrV5214*) survived challenge. We repeated this experiment with the same results (data not shown).

Mice immunized with *lcrV*-expressing strains χ 9558(pYA4665) (*lcrV5214*) and χ 9558(pYA3841) (*lcrV196*), or with strain χ 9558(pYA3996) (*psn*) were significantly protected against intranasal *Y. pestis* challenge ($P<0.05$), although this protection was not as great as that achieved by injection with purified LcrV ($P<0.01$). All mice inoculated with either BSG or control strain χ 9558(pYA3620) succumbed to the challenge.

4. Discussion

LcrV is a key component of the type III secretion system in *Y. pestis* and is required for delivery of effector proteins into host cells [40]. LcrV is also a protective antigen. Mice immunized with LcrV are protected against *Y. pestis* challenge [43]. In addition, antibodies against LcrV are protective, as passive transfer of sera from LcrV-immunized rabbits to naïve mice provides a high degree of protection against *Y. pestis* challenge [44]. Protective epitopes are located in the central region of LcrV (residues 135 to 275) [45]. Moreover, deletion of 30 aa near the C-terminal end of LcrV (aa 271–300) seems to have no impact on

protective efficacy [46]. Furthermore, Nakajima and Brubaker [47] suggest that LcrV may be immunosuppressive by inhibiting cytokine production. LcrV is able to suppress TNF- α production by inducing expression of IL-10 [9]. Residues 31–57 are involved in suppression of the innate immune response by interaction with the TLR2 receptor. When lysine residue 42 of the *lcrV* gene of *Y. enterocolitica* strain WA-C (pYV) was replaced by a glutamine residue, the ability of the mutant LcrV to interact with TLR2 was abolished [12]. In addition, Abramov *et al.* [6] performed some elegant studies to determine which residues of LcrV were important for interaction with TLR2. They utilized peptides consisting of aa 31–50 or 193–210 of LcrV. Both peptides bound to TLR2 receptors with strong affinity ($K_d = 10^{-10}$ M). The aa 31–50 peptide encodes LEEL₃₂₋₃₅ and DKN₄₁₋₄₃, two motifs required for the biological activity of LcrV. The aa 193–210 peptide encodes two similar motifs, DEEL₂₀₃₋₂₀₆ and DKN₁₈₅₋₁₈₇. An E34Q LcrV mutation reduced the affinity between the aa 31–50 peptide and TLR2 by three orders of magnitude (1,000-fold increase in K_d) and a deletion of DEEL₂₀₃₋₂₀₆ in the aa 193–210 peptide completely abolished its binding to TLR2 ($K_d > 10^{-3}$ M). They also demonstrated that the DKN motifs directed high affinity binding to CD14. These authors also observed that LcrV binds to receptor bound human IFN- γ , but not mouse IFN- γ . Thus, modification or deletion of these residues may permit elimination of LcrV sequences important in modulating the host immune system to obtain a safer, more immunogenic vaccine. However, others have called into question the importance of the LcrV-TLR2 interaction in mouse models of plague [7,48].

Based on the above studies, we designed plasmids directing synthesis of: (a) LcrV₁₉₆, a fragment of LcrV (131–327) that contributes to protection but lacks the LEEL₃₂₋₃₅ and DKN₄₁₋₄₃ sequences and (b) LcrV₅₂₁₄ (K42Q, E34Q, E35Q, E204Q, E205Q) in which both the LEEL₃₂₋₃₅ and DEEL₂₀₃₋₂₀₆ sites are altered, in addition to introduction of the K42Q mutation. After codon optimization to enhance the expression, *lcrV196* and *lcrV5214* were cloned into the β -lactamase secretion vector (pYA3620). As previously observed with this secretion system [31,41], both mutant proteins were secreted into the supernatant, mimicking the natural expression of LcrV.

All mice orally immunized by RASV-LcrV₁₉₆ and RASV-LcrV₅₂₁₄ developed high serum IgG titers against rLcrV. The truncated protein was sufficient to afford a full protection against subcutaneous challenge, confirming that the central region of the protein contains the principal epitopes involved in protection. In addition, the LcrV₅₂₁₄ protein delivered by RASV also elicited a strong humoral response (Fig. 3) and provided significant protection against intranasal challenge, but no protection against subcutaneous challenge (Table 3). This lack of protection was surprising, as we anticipated that substitution of amino acid residues reported to be involved in immunosuppression would lead to greater protective immunogenicity. The reason for the lack of protection observed with LcrV₅₂₁₄ is unlikely to be related to improper folding of the mutant protein. We have constructed a *Y. pestis* mutant strain in which we substituted the native *lcrV* gene with a gene that encodes the same 5 amino acid substitutions present in *lcrV5214*, but was not codon-optimized for synthesis in *Salmonella*. That strain is identical to its wild-type parent in its ability to direct LcrV and Yop secretion (unpublished observations), a key function of LcrV. Based on our results, we conclude that LcrV₅₂₁₄ is not suitable for use in a vaccine to generate protective immunity against plague.

Psn, the outer membrane receptor for the siderophore yersiniabactin, is an integral component of an inorganic iron transport system that functions at 37°C [42]. The entire *psn* gene was cloned without codon optimization to avoid the toxicity we often encounter when outer membrane proteins are synthesized in RASV. Immunization with RASVs synthesizing Psn elicited a protective response against bubonic plague (Tables 2,3). It was previously shown that *Y. pestis* strains with a deletion of *psn* are avirulent by a subcutaneous route of

infection [18]. Recent studies have demonstrated that *psn* is also required for full virulence of *Y. pestis* by a pneumonic route of infection [19]. Our results showing partial, but significant protection against pneumonic plague confirms that *Psn* plays a role during the development of pneumonic plague. This observation is consistent with the fact that *psn* transcription is up-regulated in the lungs of infected mice [49].

We have observed previously the ability of χ 8501 harboring an empty vector (e.g. χ 8501(pYA3620)) to elicit cross-protective immunity against *Y. pestis* challenge in immunized mice [26] and confirmed that observation in this study (Table 2). Interestingly, χ 9558(pYA3620) was ineffective at inducing any cross-protective immunity to plague challenge (Table 3). This might be related to the reduced levels of colonization observed for χ 9558 derivatives (Fig. 2). In spite of this apparent deficiency, *Salmonella* strain χ 9558 was an effective delivery vehicle for *Psn* and *LcrV*196, inducing significant protective immunity against both subcutaneous and intranasal challenges (Table 3). χ 9558(pYA3996) (*psn*) was slightly more effective at inducing protective immunity against both challenge routes than χ 8501(pYA3996) (Tables 2,3). The ability of χ 9558 to effectively deliver *Yersinia* antigens, coupled with its overall improved safety profile [27,32], indicates that the strategy used to construct χ 9558 provides a safe and effective delivery vector for plague antigens. *S. Typhi* strains with a genotype similar to χ 9558 have been constructed for evaluation in human volunteers [50].

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Figure 1.

Evaluation of LcrV, HmuR and Psn synthesis in RASV. (A) The presence of LcrV, HmuR and Psn was observed in whole cells separated on Coomassie brilliant blue-stained SDS-polyacrylamide gels. Lanes: (1) χ 8501(pYA3342), (2) χ 8501(pYA3995) (*hmuR*), (3) χ 8501(pYA3996) (*psn*), (4) χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT), (5) χ 9558(pYA3342), (6) χ 9558(pYA3996) (*psn*), (7) χ 9558(pYA3841) (*bla*-SS-*lcrV*196-CT), (8) χ 9558(pYA4661) (*bla*-SS-*lcrV*-CT), (9) χ 9558(pYA4665) (*bla*-SS-*lcrV*5214-CT). MW: molecular mass markers. Asterisks appearing on the right of each lane denote the expected size of the *Y. pestis* protein synthesized by that vaccine strain. Western blots were probed with anti-LcrV (B), anti-HmuR (C) or anti-Psn rabbit antibodies (D). Whole cell lysates, culture supernatants (B) and outer membrane fractions (C) and (D) were examined. Lanes for B: (1–6) whole cells, (7–10) culture supernatants, MW: molecular mass markers (1) χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT), (2) χ 9558(pYA3841) (*bla*-SS-*lcrV*196-CT), (3) χ 9558(pYA4661) (*bla*-SS-*lcrV*-CT), (4) χ 9558(pYA4665) (*bla*-SS-*lcrV*5214-CT) (5) χ 9558(pYA3342), (6) χ 8501(pYA3342), (7) χ 9558(pYA4665) (*bla*-SS-*lcrV*5214-CT), (8) χ 9558(pYA4661) (*bla*-SS-*lcrV*-CT), (9) χ 9558(pYA3841) (*bla*-SS-*lcrV*196-CT), (10) χ 9558(pYA3620). The expected sizes of the antigens made by each vaccine strain are: 23.5 kDa (LcrV196), 29 kDa (Bla-SS-LcrV196-CT), 37.2 kDa (LcrV and LcrV5214) and 42.7 kDa (Bla-SS-LcrV-CT and Bla-SS-LcrV5214-CT). Lanes for C: (1–2) whole cells, (3–4) outer membrane fractions, (1) χ 8501(pYA3995) (*hmuR*), (2) χ 8501(pYA3342), (3) χ 8501(pYA3995) (*hmuR*) (4) χ 8501(pYA3342). MW: molecular mass markers. Expected size of HmuR is 74.1 kDa. Lanes for D: (1,2,5,6) whole cells, (3,4,7,8) outer membrane fractions, MW: molecular mass markers (1) χ 8501(pYA3396) (*psn*), (2) χ 8501(pYA3342), (3) χ 8501(pYA3396) (*psn*), (4) χ 8501(pYA3342), (5) χ 9558(pYA3396) (*psn*), (6) χ 9558(pYA3342), (7) χ 9558 (pYA3396) (*psn*), (8) χ 9558 (pYA3342). Expected size of Psn is 73.7 kDa.

Figure 2. Persistence of *S. Typhimurium* strains χ 8501(pYA3841) (*lcrV196*), χ 8501(pYA3995) (*hmuR*), χ 8501(pYA3996) (*psn*), χ 8501(pYA3332), χ 9558 (pYA3841) (*lcrV196*), χ 9558(pYA4665) (*lcrV5214*), and χ 9558(pYA3620) in Peyer's patches (A), spleen (B) and liver (C) of orally immunized mice. Results are expressed as the average number of CFU recovered from the tissues of 3 mice at days 3, 7, 10 and 15.

Figure 3. Serum IgG responses in mice orally immunized with RASV expressing *Y. pestis* antigens. Mice were orally immunized with attenuated *S. Typhimurium* strains χ 8501(pYA3841) (*lcrV196*), χ 9558(pYA3841), χ 9558(pYA4665) (*lcrV5214*), χ 8501(pYA3996) (*psn*),

χ 9558(pYA3996), or χ 8501(pYA3995) (*hmuR*). Strains χ 8501(pYA3342) and χ 9558(pYA3620) that do not express a *Y. pestis* antigen served as negative controls. Panels: (A) total anti-LcrV IgG, or anti-Psn IgG (IgGt), (B) total anti-LPS IgG, (C) anti-LcrV IgG₁ or anti-Psn IgG₁, (D) anti-LcrV IgG_{2a} or anti-Psn IgG_{2a}, (E) total anti-HmuR IgG. The HmuR ELISA was performed using a 1:100 dilution of pooled sera.

Table 1

Strains and plasmids used in this study

Strain or Plasmid	Genotype or relevant characteristics	Derivation or source
Strains		
<i>E. coli</i>		
DH5α	F ⁻ ϕ80 <i>lacZΔ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17 (r_K⁻ m_K⁺) phoA glnV44 thi-1 gyrA96 relA1 λ⁻</i>	Invitrogen
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B (r_B⁻ m_B⁻) λDE3 [lacI lacUV5 -T7 gene 1 ind1 sam7 nin5]</i>	Stratagene
χ6212	F ⁻ λ ⁻ ϕ 80 <i>Δ(lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4</i>	[37]
<i>Salmonella</i>		
χ8501	<i>hisG Δcrp-28 ΔasdA16</i>	[37]
χ9558	<i>Δpmi-2426 Δ(gmd-fcl)-26 ΔP_{fur81}::TT araC P_{BAD} fur Δ P_{crp527}::TT araC P_{BAD} crp Δ asdA27::TT araC P_{BAD} c2 Δ araE25 ΔaraBAD23 ΔrelA198::araC P_{BAD} lacI TT ΔsopB1925 ΔagfBAC811</i>	[29,32]
<i>Y. pestis</i>		
CO92	Pgm+ Lcr+ Pst/Pla ^a	[33]
KIM6+	Pgm ⁺ , pMT1, pPCP1, cured of pCD1	[35]
Plasmids		
pJIT7	pBR322, the 3.6 kb HindIII-G fragment from pCD1 encoding <i>lcrGVH</i> .	[36]
pYA3332	Asd ⁺ ; p15A <i>ori</i>	Lab collection
pYA3342	Asd ⁺ ; pBR <i>ori</i>	[37]
pYA3620	Asd ⁺ ; pBR <i>ori</i> ; β-lactamase signal sequence-based periplasmic secretion	[51]
pYA3841	pYA3620 <i>lcrV196</i> (codon optimized)	[26]
pYA4665	pYA3620 <i>lcrV5214</i>	This study
pYA3995	pYA3332 <i>hmuR</i>	This study
pYA3996	pYA3332 <i>psn</i>	[26]

^a Pgm+ - possesses 102-Kb pigmentation locus encoding the Yersiniabactin system and the Hms-dependent biofilm system; Lcr+ - possesses pCD1 plasmid encoding T3SS system, effector Yops, and LcrV; Pst/Pla+ - possesses pPCP1 plasmid encoding the bacteriocin pesticin and the virulence factor plasminogen activator, Pla

Survival of mice immunized with RASV χ 8501 harboring different plasmids after *Y. pestis* challenge^a.

TABLE 2

	Subcutaneous challenge		Survivors/total		Intranasal challenge		Survivors/total
Challenge dose (CFU)	60	1300			443	4450	
χ 8501(pYA3841)(<i>crV</i> 196)	8/8	8/8		16/16 ^{**}	5/8	3/8	8/16 [*]
χ 8501(pYA3996)(<i>psn</i>)	4/8	6/8		10/16 [*]	3/8	0/8	3/16
χ 8501(pYA3995)(<i>hmuR</i>)	3/8	2/8		5/16	0/8	0/8	0/16
χ 8501(pYA3342)	3/8	2/8		5/16	0/8	0/8	0/16
BSG control	0/4	-		0/4	0/4	-	0/4

^a Animals were vaccinated twice at 10 day intervals and challenged with *Y. pestis* CO92 28 days after the last immunization.

^b Number of surviving animals at 14 days after challenge/number of animals challenged.

^c The LD₅₀ of CO92 by the subcutaneous route in non-immunized BALB/c mice is less than 10 CFU (data not shown).

^d The LD₅₀ of CO92 by the intranasal route in non-immunized BALB/c mice is approximately 100 CFU (data not shown).

* $P < 0.05$.

** $P < 0.01$ compared to the BSG group.

TABLE 3
Survival of mice immunized with RASV γ 9558 harboring different plasmids after *Y. pestis* challenge^a.

	Subcutaneous challenge ^b		Survivors/total	Intranasal challenge ^{b,c}	Survivors/total
Challenge dose (CFU)	450	5630		4100	44000
γ 9558(pYA3841)(<i>lcrV</i> 196)	8/8 ^d	7/8 ^d	15/16 ^{**d}	2/8	4/8
γ 9558(pYA4665)(<i>lcrV</i> 5214)	0/8 ^d	0/8 ^d	0/16 ^d	5/8	1/8
γ 9558(pYA3996)(<i>pnn</i>)	7/8	6/8	13/16 ^{**}	4/8	3/8
γ 9558(pYA3620)	0/8	0/8	0/16	0/8	0/8
LcrV protein	4/4 ^d	4/4 ^d	8/8 ^{d**}	4/4	2/4
BSG control	0/4	0/3	0/7	0/3	0/1

^a Animals were vaccinated twice at 10 day intervals and challenged with *Y. pestis* CO92 28 days after the last immunization.

^b Number of surviving animals at 14 days after challenge/number of animals challenged.

^c The LD50 of CO92 by intranasal route in non immunized BALB/c mice is ~100 CFU (data not shown).

^d The challenge doses in these experiments were 380 and 3800 CFU.

* $P < 0.05$,

** $P < 0.01$ compared to the BSG group.