Published in final edited form as:

Vaccine. 2009 August 27; 27(39): 5363–5370. doi:10.1016/j.vaccine.2009.06.078.

Oral Vaccination with LcrV from *Yersinia pestis* KIM delivered by live attenuated *Salmonella enterica* serovar Typhimurium elicits a protective immune response against challenge with *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*

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Abstract

The use of live recombinant attenuated *Salmonella* vaccines (RASV) synthesizing *Yersinia* proteins is a promising approach for controlling infection by *Yersinia* species. In this study, we constructed attenuated *Salmonella* strains which synthesize a truncated form of LcrV, LcrV196 and evaluated the immune response and protective efficacy elicited by these strains in mice against two other major species of *Yersinia*: *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. Surprisingly, we found that the RASV strain alone was sufficient to afford nearly full protection against challenge with *Y. pseudotuberculosis*, indicating the likelihood that *Salmonella* produces immunogenic cross-protective antigens. In contrast, *lcrV196* expression was required for protection against challenge with *Y. enterocolitica*. As LcrV has been previously shown to provide protection against *Yersinia pestis*, these findings indicate that a single vaccine may provide protection against the three human pathogenic species of *Yersinia*.

Keywords

Recombinant Attenuated *Salmonella* Vaccine; LcrV; *Yersinia*

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. *Y. enterocolitica* and *Y. pseudotuberculosis* are food-borne pathogens found widely in the environment and are a common cause of animal infections, affecting several mammalian and avian species [3–5]. In humans, the infections are initiated by consumption of contaminated food or water leading to gastrointestinal symptoms ranging from diarrhea to acute mesenteric

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lymphadenitis [2,6]. After oral ingestion, the bacteria move to the small intestine and invade the intestinal epithelium via interactions with the specialized M cells overlying the Peyer's patches. This interaction is mediated by the *inv* and *ail* genes [7,8]. Once established, they colonize the underlying lymph tissues [9]. Occasionally, *Y. enterocolitica* but not *Y. pseudotuberculosis* may spread to the spleen and liver, causing systemic disease [6].

Strong molecular evidence supports the fact that *Y. pestis* recently diverged from *Y. pseudotuberculosis* and is therefore closely related [10]. *Y. enterocolitica* has evolved into a more heterogeneous group, classified into 6 biogroups [11,12] including biotype 1B, associated with human infection. Biotype 1B includes the most virulent serotype O8, primarily isolated in North America [2,13].

The three human pathogenic *Yersinia* species harbor the 70-kb low calcium response plasmid that encodes essential virulence factors including the type III secretion system and the adherence protein YadA [14,15] (not functional in *Y. pestis* [16]). LcrV is a secreted, multifunctional protein that is central to the activity of the type III secretion system apparatus. LcrV affects 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 [17]. LcrV is highly immunogenic, inducing a protective immune response, and is therefore a component of most plague vaccines [18]. It is highly conserved among the *Yersinia* species, although some protein polymorphism has been described which is especially evident in *Y. enterocolitica* strains [19,20]. As a result of this polymorphism, there are two distinct types of LcrV, LcrV-YenO8, derived from *Y. enterocolitica* serotype O8 and LcrV-Yps, which includes LcrV from *Y. pestis*, *Y. pseudotuberculosis* and other *Y. enterocolitica* serotypes [19]. While the two LcrV types differ at a number of amino acids across the protein, the most notable difference lies in a hypervariable region between aa 225 and 232 that includes a 9 aa insertion in the LcrV-YenO8 group. One exception is the O8 strain *Y. enterocolitica* 8081, which synthesizes an LcrV protein that is more like LcrV-Yps across aa 225–232 [19]. Monospecific antisera against the two types of LcrV used in passive immunization studies, protected mice against challenge with strains expressing the homologous LcrV, but not strains expressing LcrV from the other group [19]. In addition, LcrV contributes to pathogenicity via TLR2-mediated IL-10 induction [21]: LcrV-YenO8 of *Y. enterocolitica* causes TNF- α suppression in macrophages in a CD14 and TLR2-dependant manner by inducing IL-10 [22–24]. However, the TLR2-LcrV interaction seems to play less of a role in *Y. pestis* and *Y. pseudotuberculosis* pathogenicity [25–27]. Administration of TNF- α and INF- γ can promote survival after *Y. pestis* challenge [28].

Live attenuated *Salmonella* strains were first developed as vaccines to prevent disease caused by *Salmonella* infections of both humans and animals [29,30]. 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 that is 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.

Ongoing research in our laboratory has been directed toward improving the immunogenicity and stability of *Salmonella* delivery strains and methods of antigen expression. To maintain the plasmid expressing an antigen of interest and avoid the use of antibiotics, a balanced-lethal host-vector system has been designed. In this system, the gene encoding aspartate β -semialdehyde dehydrogenase (*asd*) is used to maintain plasmids co-expressing the protective antigens in Δ *asd* *Salmonella* strains [31,32]. We have shown previously that secretion of a protective pneumococcal antigen, PspA, to the periplasm of an attenuated *Salmonella* strain enhances the immune response and protective efficacy of the vaccine compared to a strain that

expresses, but does not secrete PspA [33]. We have designed a number of secretion vectors that rely on fusing an antigen of interest to secretion signals from β -lactamase, including pYA3493, which is designed to enable an amino-terminal fusion between the first 35 aa of the β -lactamase gene, *bla*, (*bla*-SS) and an antigen of interest [34] and pYA3620, which in addition to the amino-terminal *bla*-SS fusion creates a C-terminal fusion with the C-terminal 22 aa of *bla* (*bla*-SS-CT) [35,36]. The β -lactamase C-terminus is required for secretion of native β -lactamase [37], and, in some cases, can significantly improve heterologous antigen secretion [35,36].

Immunization of mice with attenuated *Salmonella* expressing a β -lactamase signal sequence fusion to the truncated *lcrV* gene, *lcrV196*, induced a strong serum IgG response against LcrV and protected the immunized mice against challenge with virulent *Y. pestis* [38]. The purpose of the present study is to investigate the immunogenicity and protective efficacy of orally administered recombinant attenuated *Salmonella* vaccines (RASV) synthesizing the *Y. pestis* LcrV196 protein against challenge with virulent *Y. pseudotuberculosis* and *Y. enterocolitica*. In addition, we compared the effects of two different signal sequence fusion proteins that direct the antigen to the periplasmic space and supernatant with a construct that resulted in cytoplasmic expression.

2. Materials and methods

2.1 Bacterial Strains and growth media

Y. enterocolitica strain 8081, serotype O8, was kindly provided by V. Miller. *Y. pseudotuberculosis* PB1/+ and *Y. enterocolitica* strain WA were kindly provided by R. Perry. *Yersinia* strains were routinely grown in heart infusion broth (Difco, Detroit, MI) at 28°C.

Salmonella enterica serovar Typhimurium strain χ 8501 (*hisG* Δ *crp*-28 Δ *asdA16*) was used as the vaccine vector in all studies [34]. *Escherichia coli* strain χ 6212 ($F^- \lambda^- \phi 80 \Delta(lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 \Delta asdA4$) [34] was used for cloning. *E. coli* strain BL21(DE3) ($F^- ompT hsdS_B (r_B^- m_B^-) gal dcm\lambda$ (DE3)) was used for expressing proteins for purification. Bacteria were grown in LB broth [39], or on LB agar or MacConkey agar supplemented with 1% lactose. Diaminopimelic acid (DAP) was included in the growth media at 100 μ g/ml when necessary for growth of non-complemented *asd* strains and for plasmid stability tests.

2.2 Construction of plasmids pYA3839, pYA3840 and pYA3841

As a first step in vaccine construction, the *lcrV* DNA sequence obtained from *Y. pestis* KIM was codon-optimized for expression in *Salmonella*. We identified codons in *lcrV* that are used with less than a 2% frequency in highly expressed *Salmonella* genes [40]. These codons were then modified to correspond to more frequently used codons in *Salmonella*. The codon-modified truncated *lcrV* sequence, corresponding to aa131-aa326 (*lcrV196*) cloned in pUC19, was previously described [38].

For construction of pYA3839 a 616 bp PCR fragment encoding the codon-optimized, *lcrV196* sequence was cloned into *Asd*⁺ plasmid pYA3342 [34]. The primers used for PCR were 5'-GACCATGGGAATCGATGATGATATTTTGAAAGTG and 5'-CCCAAGCTTTCATTACCAGACGTGTCATCGAG. These primers also encode an *Nco*I or *Hind*III site (underlined), respectively. The *lcrV196* gene was amplified using pUC19-*lcrV196* as the template, digested with *Nco*I and *Hind*III, and ligated into the corresponding sites of pYA3342.

A similar strategy was used to clone codon-optimized *lcrV196* into the *Asd*⁺ secretion vector pYA3493 [34] to create pYA3840 using primers

GCTCTAGAGAATTCATCGATGATGATATTTTGAAAGTG and CCCAAGCTTTCATTACCAGACGTGTCATCGAG, which contain EcoRI and HindIII sites, respectively (underlined). The PCR product amplified from pUC19-*lcrV196*, was digested with EcoRI and HindIII and the 613-bp fragment was ligated into the EcoRI and HindIII sites of plasmid pYA3493. This created an in-frame fusion between the first 35 aa of β -lactamase and the amino-terminal amino acid of LcrV196, creating *bla*-SS-*lcrV196*. Of the 35 aa derived from β -lactamase, the first 23 aa constitute a *sec*-dependent signal sequence. Upon secretion, this sequence is cleaved, leaving the first 12 aa of mature β -lactamase fused to LcrV196.

The construction of plasmid pYA3841 was described previously [38]. This plasmid encodes the same *bla*-SS-*lcrV196* fusion as pYA3840 and also contains the C-terminal 22 amino acids of β -lactamase fused to the C-terminus of LcrV196, creating *bla*-SS-*lcrV196*-CT.

All three plasmids were electroporated into *Salmonella* strain χ 8501. The DNA sequence of the *lcrV196* inserts in each was confirmed to be correct by DNA sequence analysis (Arizona State University, USA). Plasmid stability in strain χ 8501 was performed as previously described [34].

2.3 SDS PAGE and western blot analysis

To evaluate antigen expression, *Salmonella* strain χ 8501 harboring either pYA3342, pYA3493, pYA3620, pYA3839, pYA3840 or pYA3841 was cultured in LB broth at 37°C; *Y. pestis* KIM, *Y. pestis* CO92, *Y. pseudotuberculosis* PB1/+, *Y. enterocolitica* 8081 were cultured in HIB broth at 28°C. The cultures were harvested when the cultures reached an optical density at 600 nm (OD₆₀₀) of 0.8. The bacteria were harvested by centrifugation at 10,000 $\times$ g and the pellets resuspended in Laemmli sample buffer containing 2% 2-mercaptoethanol. To evaluate secreted proteins, culture supernatants were passed through a 0.22 μ m filter and precipitated overnight with 20% TCA (v/v). After centrifugation, the TCA pellet was resuspended in cold PBS, and acetone precipitated. The precipitates were washed with acetone, resuspended in PBS and stored at -20°C.

The proteins were separated by SDS-polyacrylamide gel electrophoresis as previously described [41] 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 followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). In some experiments sera from mice immunized with a *S. Typhimurium* vaccine strain (χ 8501) was used as the primary antibody followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). Antibody complexes were detected with the NBT-BCIP liquid substrate (Amresco). The experiments were performed three times.

2.4 Immunization of mice and challenge studies

Female Swiss Webster mice, 6–8 weeks of age, were purchased from Charles River (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 to an OD₆₀₀ of 0.9 and concentrated to 5 $\times$ 10¹⁰ CFU/ml in phosphate-buffered saline containing 0.01% gelatin (BSG)[42]. Groups of mice were orally immunized with 20 μ l of RASV suspensions on days 0 and 10. Challenge was performed 28 days after the second immunization (day 38). For oral challenge, a freshly prepared culture of *Y. pseudotuberculosis* strain pB1/+, *Y. enterocolitica* 8081 or WA grown in heart infusion broth (HIB) at 28°C was used. Blood samples were collected on days 0, 21, 32, and at the end of the experiment. Mice were observed daily and

mortality was recorded for 35 days after the challenge. The surviving animals were euthanized at the end of the experiment to obtain blood samples for serological analysis. The challenge experiments were performed twice, except for challenge with *Y. enterocolitica* WA, which was performed once.

2.5 Colonization of mouse tissues

Mice were immunized with $1 - 2 \times 10^9$ CFU of $\chi 8501$ (pYA3620) (control), or one of the three strains expressing *lcrV196*; $\chi 8501$ (pYA3839) (*lcrV196*), $\chi 8501$ (pYA3840) (*bla*-SS-*lcrV196*) or $\chi 8501$ (pYA3841) (*bla*-SS-*lcrV196*-CT). Three mice from each group were euthanized by asphyxiation with CO₂ at day 3, 7, 10 and 15. The Peyer's patches, spleen and part of the liver were aseptically taken from each mouse and homogenized in PBS using a PowerGen 125 S1 homogenizer (Fisher 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 from each organ. This experiment was performed twice.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc, Rochester, NY, USA) were coated with purified His₆-tagged full length LcrV (500ng/well) or *S. Typhimurium* LPS (100ng/well) (Sigma, L2262) and incubated overnight at 4°C. The expression and purification of His₆-tagged LcrV encoded by plasmid pHT-V has been previously described [43]. 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. Pooled sera from the mice in each group were diluted in PBS and added to the plates with a dilution of 1:800, except for the IgG1 analysis, where the dilution was 1:400. After 1 h incubation at 37°C, the plates were washed and incubated for 1 h at 37°C sequentially with biotin-conjugated goat anti-mouse IgG(H+L), anti-mouse IgG1, anti-mouse IgG2_a, (SouthernBiotech) (1:5000) and streptavidin conjugated to alkaline phosphatase (SouthernBiotech) (1:4000) with washings in between. Plates were then incubated with the chromogenic substrate, *p*-nitrophenyl phosphate (Sigma), for 10 min and the reaction was stopped by the addition of 2 M H₂SO₄. The optical density at 405 nm was measured with a Labsystems Multiskan MCC/340. The experiment was performed twice.

2.7 Statistical analysis

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

3. Results

3.1 Expression of *lcrV196* in RASV

Expression of *lcrV196* was evaluated in each RASV $\chi 8501$ strain by Coomassie brilliant blue-staining of SDS-PAGE gels and by western blot analysis (Fig. 1). RASV strains $\chi 8501$ (pYA3839), $\chi 8501$ (pYA3840), $\chi 8501$ (pYA3841) produced similar amounts of cell-associated LcrV (Fig. 1A, 1B). Strains carrying the empty vector plasmids did not produce any protein that reacted with anti-LcrV antibody (Fig. 1B). Plasmids pYA3840 and pYA3841 encode *bla*-SS-*lcrV196* and *bla*-SS-*lcrV196*-CT fusions that we expect will direct LcrV to the periplasm and supernatant. The supernatant fractions from $\chi 8501$ (pYA3840) and $\chi 8501$ (pYA3841) contained protein that reacted with the anti-LcrV antibody (Fig. 1C), indicating that the β -lactamase signal sequence was directing antigen secretion as expected. Supernatant fractions from strains carrying empty vector plasmids or plasmid pYA3839 in which *lcrV* is

not fused to a secretion signal sequence, did not react with the anti-LcrV antibody (Fig. 1C and data not shown).

3.2 Colonization of mice by attenuated *Salmonella* vaccine strains

Attenuated *S. Typhimurium* strain χ 8501 carrying the empty vector plasmid pYA3620 or pYA3841 encoding *bla*-SS-*lcrV*196-CT, was evaluated for the ability to colonize mouse spleen, liver and Peyer's patches (Fig. 2). We did not observe any significant differences in colonization between strains. Similar results were obtained with χ 8501(pYA3839) (*lcrV*196) and χ 8501(pYA3840) (*bla*-SS-*lcrV*196). (data not shown).

3.3 Anti-LcrV and anti-*Salmonella* LPS body responses in immunized mice

The serum IgG responses from immunized mice were analyzed by ELISA against the recombinant full length LcrV protein (Fig. 3A). No anti-LcrV IgG was detected in mice receiving the control strain χ 8501(pYA3342). Mice orally immunized with strains expressing *lcrV*196, χ 8501(pYA3839)(*lcrV*196), χ 8501(pYA3840) (*bla*-SS-*lcrV*196) or χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT), developed a strong immune response against LcrV. The anti-rLcrV response was not detected until after the second immunization (day 10) and increased over time until week 9, when the levels of anti-LcrV IgG dropped slightly. All the immunized mice developed a strong serum IgG response against *S. Typhimurium* LPS with no significant differences between groups (Fig. 3B).

We also evaluated sera for anti-LcrV IgG isotypes IgG1 (Fig. 3C) and IgG2_a (Fig. 3D). The levels of IgG2_a were greater than IgG1 levels at all time points, indicating induction of a predominantly Th1 response by all strains expressing *lcrV*196. Taken together, these results indicate that all three strains expressing *lcrV*196 induce similar serum IgG immune responses.

3.4 Protection of mice immunized against *Y. pseudotuberculosis* challenge

After our initial evaluation of serum IgG responses, we chose one strain, χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT) for further analysis. In two separate experiments, we immunized groups of 10 mice with either χ 8501(pYA3620) (control) or χ 8501(pYA3841). Mice were boosted on day 10 and orally challenged 28 days later with either 2.3×10^7 CFU (5 mice) or 2.5×10^8 CFU (5 mice) of *Y. pseudotuberculosis* PB1/+ in the first experiment and 1.8×10^8 CFU or 2.0×10^9 CFU in the second experiment. Mortality was recorded for 35 days after challenge. In preliminary experiments we determined that the oral LD₅₀ of *Y. pseudotuberculosis* PB1/+ is 1×10^7 CFU (data not shown).

Our results in the first experiment (Table 1) showed that all mice immunized with the strain expressing *lcrV*196, χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT) were protected against challenge at both doses, while only 2 of 5 and 1 of 5 mice receiving BSG buffer alone survived challenge with low or high doses of *Y. pseudotuberculosis*, respectively. Surprisingly, 4 of 5 mice given χ 8501(pYA3620), which does not express *lcrV*196 and did not elicit an immune response against LcrV (Fig. 3) were protected in both the low and high-dose challenge groups. When we repeated the experiment, all 10 non-immunized mice succumbed to challenge, while all the mice immunized with either χ 8501(pYA3620) or χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT) survived, regardless of the challenge dose (Table 1). These results indicate that vaccination with attenuated *Salmonella* strain χ 8501 is sufficient to provide significant protection against *Y. pseudotuberculosis* challenge ($P < 0.05$), regardless of whether it synthesizes LcrV.

3.5 Serum antibodies against *S. Typhimurium* proteins that cross-react with *Yersinia* proteins

Based on the above results, it is likely that *S. Typhimurium* strain χ 8501 produces one or more antigens that induce antibodies that are cross-reactive with *Y. pseudotuberculosis*. To evaluate whether *Salmonella* produces a cross-reactive protein, we performed western blot on whole cell extracts from two strains of *Y. pestis*, one strain of each of *Y. pseudotuberculosis* and *Y. enterocolitica* and the *S. Typhimurium* vaccine vector strain χ 8501. Membranes were probed with anti-sera obtained from mice immunized with the control strain χ 8501(pYA3620). The resulting western blot revealed a number of bands, including ones at 17 kDa, 25 kDa and 65 kDa that reacted strongly with the serum (Fig. 4).

3.6 Efficacy of χ 8501(pYA3841) (bla-SS-lcrV196-CT) against challenge with *Y. enterocolitica*

We also evaluated our vaccine for the ability to protect against another important enteric species, *Y. enterocolitica*. Since we had observed significant cross-protection against *Y. pseudotuberculosis* using a dose as high as 100 LD₅₀s, we decided to use an equally high dose to evaluate protection against *Y. enterocolitica*, as this would allow us to test the limits of protection provided by the vaccine and maximize the differences between groups immunized with χ 8501(pYA3841), expressing lcrV196 and groups immunized with *Salmonella* alone. Groups of five mice were orally immunized on days 0 and 10, orally challenged with 1.3×10^9 CFU of *Y. enterocolitica* 8081 and monitored for 24 days. The oral LD₅₀ of *Y. enterocolitica* 8081 is 1×10^7 CFU. We monitored the serum immune response and found that the levels of anti-LcrV IgG in mice immunized with χ 8501(pYA3342) (negative control strain used in this experiment) or χ 8501(pYA3841) was similar to what we observed in our previous experiments (Fig. 3 and data not shown). Sixty per cent of mice immunized with χ 8501 (pYA3841), the strain synthesizing LcrV196, survived challenge, while only 20% of the mice immunized with the empty vector strain χ 8501(pYA3620) were protected against *Y. enterocolitica* strain 8081 (Fig 5). All of the BSG-immunized mice succumbed to challenge. These results indicate that the vaccine expressing *lcrV196* elicited a protective immune response and, unlike the results for *Y. pseudotuberculosis*, LcrV synthesis was required to achieve significant protection against *Y. enterocolitica* challenge ($P < 0.05$).

To evaluate the effect of our vaccine to protect against an O8 strain synthesizing LcrV-YenO8, we orally challenged 2 groups of 5 immunized mice and 5 control mice with 6.9×10^8 CFU of *Y. enterocolitica* WA, which has an oral LD₅₀ of 6.4×10^6 CFU. All of the mock-immunized mice succumbed to challenge. Forty per cent of the mice immunized with χ 8501(pYA3841), survived challenge, while 20% of mice immunized with the empty vector strain χ 8501 (pYA3620) were protected. The level of protection in immunized mice was not significantly different from the controls ($P > 0.05$).

4. Discussion

LcrV has been shown to be a protective antigen against *Y. pseudotuberculosis* and *Y. enterocolitica* challenge [19,44,45]. In a recent study, mice immunized intranasally with *Lactobacillus lactis* engineered to secrete LcrV derived from *Y. pseudotuberculosis* were protected against both oral and systemic challenges, although no protection against *Y. pestis* challenge was observed [46]. In previous work, we showed that immunization of mice with χ 8501(pYA3841), an attenuated *S. Typhimurium* strain expressing *lcrV196*, protected mice against *Y. pestis* challenge [43]. In this work, we demonstrate that this same strain can induce a protective immune response against the two enteric pathogens *Y. pseudotuberculosis* and *Y. enterocolitica* 8081 (Table 1 and Fig. 5). Immunized animals developed a serum IgG response against LcrV (Fig. 3) that was primarily Th1-based, as is typical for *Salmonella* vaccines, whereas animals immunized with the control strain did not produce anti-LcrV antibodies. The

immune response elicited in mice immunized with χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT) afforded significant protection against oral challenge with 100 LD₅₀s of a *Y. enterocolitica* O8 strain 8081 (Fig. 5). The fact that we used a truncated protein as the immunogen confirms that the central region of LcrV contains the principal epitope(s) needed for protection against human pathogenic *Yersiniae* as seen by others [44,47].

LcrV is polymorphic and strains expressing this antigen can be divided into 2 different groups [19]. One group consists of *Y. pestis*, *Y. pseudotuberculosis*, and the O3 serotype of *Y. enterocolitica*. The second group contains additional amino acids within the hypervariable region of LcrV and is represented by most serotype O8 strains of *Y. enterocolitica*. The *Y. enterocolitica* 8081 strain used in this study, while being serotype O8, has an LcrV more closely related to that of the O3 strains compared to other O:8 strains like WA and NTC10938 that are more divergent. Antisera against LcrV from *Y. pestis* or the *Y. enterocolitica* O3 serotype protects against *Yersiniae* expressing a related LcrV protein but not against O8 serotypes and vice versa [19,44]. Consistent with these findings, vaccine strain χ 8501(pYA3841), expressing *Y. pestis* *lcrV*, provided only a low level of protection against challenge with *Y. enterocolitica* strain WA that was not statistically significant.

However Schmidt *et al.* found that active immunization with LcrV from an O8 serotype of *Y. enterocolitica* did protect against *Y. pseudotuberculosis* infections while protection via passive immunity required higher levels of antisera [45]. A more recent analysis of the LcrV hypervariable hot spot at amino acids 41–60 provided a basis for regrouping all of the O:8 strains into a single group whereas *Y. pestis* and *Y. pseudotuberculosis* belong to another group [20]. This sequence is related directly to the level of IL-10 expression: strain 8081 belongs to the most virulent group, where TLR2-mediated up-regulation of IL-10 is the strongest [20]. Interestingly, the virulence of *Y. enterocolitica* strains in this virulent group diminishes after alteration of the TLR2 binding site, whereas no significant difference in virulence has been observed for *Y. pestis* [26]. Therefore, the IL-10 response seems play a greater role in the virulence of *Y. enterocolitica*. Based on these results, it may be necessary to remove the immunomodulatory residues of LcrV to obtain a safer and more efficient vaccine. Presumably, residues 31-57 of LcrV interact with TLR2 receptor to suppress the innate immune response [20,23]. In our studies, we used RASV expressing a fragment of the *lcrV* gene encoding the 131-327 residues, without the TLR2 interacting sequence but with the central part of the protein that contributes to protection. However, Overheim [48] and Abramov [49] observed that over sequences on LcrV in the C-terminal end of the protein could also be involved in the up-regulation of IL-10.

Since our LcrV was derived from *Y. pestis* and therefore would be expected to protect against the 8081 strain of *Y. enterocolitica*, it will be interesting to evaluate an *lcrV* mutant where this sequence has been modified to determine if it protects against other O8 serotypes of *Y. enterocolitica* including strain WA. If so, it may be possible to develop a single vaccine against all of the medically important *Yersinia*.

Perhaps the most surprising finding in this study is that mice immunized with the control strain, χ 8501(pYA3620) were fully protected against challenge with up to 100 LD₅₀s of *Y. pseudotuberculosis* (Table 1). The basis of this protection is not clear. However, we performed a similar experiment with a different attenuated *S. Typhimurium* strain that did not express a *Yersinia* antigen and that was less invasive than χ 8501, based on the strains ability to colonize Peyer's patches, spleen and liver (data not shown). In that experiment, we found that only 50% of the mice immunized with this strain were protected, indicating that colonization of lymphoid tissues is important for induction of cross-protective immunity.

Immune sera from mice immunized with the *S. Typhimurium* control strain reacted with a number of proteins from all three *Yersinia* species tested (Fig. 4). Despite this, when mice immunized with the control strain, χ 8501(pYA3620) were challenged with 100 LD₅₀s of *Y. enterocolitica* strains 8081 or WA (Fig. 5), the level of cross-protection provided by the *Salmonella* vector strain was not statistically significant ($P>0.05$), unlike what we observed for *Y. pseudotuberculosis* challenge (Table 1). There are several genetic loci present in *Y. enterocolitica* that promote intestinal colonization and survival, including the cellulose biosynthetic operon (*cel*) and the hydrogenase loci (*hyf*), that are absent from *Y. pseudotuberculosis* [12]. The *cel* operon may be involved in production of an extracellular matrix whose presence increases retention time in the gut. The hydrogenase loci provides *Y. enterocolitica* with the ability to utilize locally generated hydrogen as an energy source, a characteristic essential for gut colonization [50] and, in addition, it may serve a role in osmoprotection. The absence of these and other loci in *Y. pseudotuberculosis* could influence the enteric environment occupied by the two *Yersinia*e [12]. Exactly how these niche differences affect the cross-protective efficacy of *Salmonella* is not clear and is an interesting question for future study.

Our long-term goal is to develop a *Salmonella* vaccine strain expressing multiple *Yersinia* antigens to protect against all pathogenic *Yersinia* species. Combining the results reported here with those obtained in our previous work, we have demonstrated that a single attenuated *S. Typhimurium* strain expressing truncated *lcrV* can protect mice against challenge with *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*. The low level of protection observed in immunized mice against *Y. enterocolitica* challenge, in particular strain WA, is an important concern that will be addressed in future studies. We are currently developing new means to attenuate and enhance the immunogenicity of *Salmonella* vaccine strains and are evaluating additional *Yersinia* antigens to improve the protective efficacy of our vaccines against these important pathogens.

Acknowledgments

We thank S. Straley for providing plasmid pHT-V. J. Kilbourne is thanked for her expert assistance with animal experiments. This work was supported by National Institutes of Health grant 5R01 AI057885.

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Figure 1.

Evaluation of LcrV in whole cells and culture supernatants. The presence of LcrV was observed in whole cells separated on Coomassie brilliant blue-stained SDS-polyacrylamide gels (A). The presence of LcrV was evaluated by western blots of whole cells (B) and culture supernatants (C) probed with rabbit anti-LcrV polyclonal antibodies. Lanes: (1) χ 8501 (pYA3342), (2) χ 8501(pYA3839) (*lcrV196*), (3) χ 8501(pYA3493), (4) χ 8501(pYA3840) (*bla-SS-lcrV196*), (5) χ 8501(pYA3620), (6) χ 8501(pYA3841) (*bla-SS-lcrV196-CT*). MW: molecular mass markers. The predicted molecular weights of LcrV196 and LcrV196 fusion proteins are 23.5 kDa (pYA3839), 26.3 kDa (pYA3840) and 29 kDa (pYA3841).

Figure 2. Persistence of *S. Typhimurium* strains χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT) and χ 8501 (pYA3620) in Peyer's patches (A), spleens (B), and livers (C) of orally immunized mice. Results are expressed as individual values recovered from the tissues of each mouse at days 3, 7, 10 and 15 (3 animals per time point).

Figure 3.

Serum IgG responses in orally immunized mice. Mice were immunized with *lcrV196*-expressing attenuated *S. Typhimurium* strains χ 8501(pYA3839) (*lcrV196*), χ 8501(pYA3840) (*bla-SS-lcrV196*) and χ 8501(pYA3841) (*bla-SS-lcrV196-CT*). Strain χ 8501(pYA3342) that does not express *lcrV196* served as a negative control. Panels: (A) total anti-LcrV IgG, (B) total anti-LPS IgG, (C) anti-LcrV IgG1, (D) anti-LcrV IgG2a.

Figure 4.

Western blot performed on *Salmonella* and *Yersinia* cells. Blots were probed with mouse anti-*S. Typhimurium* strain χ 8501 sera. Lanes: (1) *Y. pestis* KIM, (2) *Y. pestis* CO92, (3) *Y. pseudotuberculosis* PB1/+, (4) *Y. enterocolitica* 8081, (5) *S. Typhimurium* χ 8501. MW: molecular mass markers.

Figure 5.

Survival of mice orally immunized with χ 8501(pYA3620) or χ 8501(pYA3841) (*bla*-SS-*lcrV*196-CT) or mice mock-immunized with BSG, followed by challenge with 1.3×10^9 CFU of *Y. enterocolitica* 8081. $P < 0.05$ for mice immunized with χ 8501(pYA3841) compared to the other two groups. This experiment was performed twice with similar results.

TABLE 1
Effect of immunization with RASVs followed by oral *Y. pseudotuberculosis* challenge^a.

	Experiment 1		Experiment 2		total
Challenge dose (CFU)	2.3×10 ⁷	2.5×10 ⁸	1.8×10 ⁸	2.0×10 ⁹	
χ8501(pYA3841) (<i>bla</i> -SS- <i>lcrV</i> 196-CT)	5/5 ^b	5/5	5/5	5/5	10/10 ^{***}
χ8501(pYA3620)	4/5	4/5	5/5	5/5	10/10 ^{***}
BSG control	2/5	1/5	0/5	0/5	0/10

^a Animals were vaccinated twice at 10 day intervals and challenged with *Yersinia pseudotuberculosis* strain pB1/+, 28 days after the last immunization.

^b Number of surviving animals at 35 days after challenge/number of animals challenged.

^c The LD50 of PB1+ in non immunized BALB/c mice is 1 × 10⁷ (data not shown).

* $P < 0.01$,

** $P < 0.05$, or

*** $P < 0.001$ compared to the BSG group.