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Oral administration of a recombinant attenuated *Yersinia pseudotuberculosis* strain elicits protective immunity against plague

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Abstract

A *Yersinia pseudotuberculosis* PB1+ (Yptb PB1+) mutant strain combined with chromosome insertion of the *caf1R-caf1A-caf1M-caf1* operon and deletions of *yopJ* and *yopK*, χ 10068 [pYV- ω 2 (*yopJ315 yopK108*) *lacZ044::caf1R-caf1M-caf1A-caf1*] was constructed. Results indicated that gene insertion and deletion did not affect the growth rate of χ 10068 compared to wild-type Yptb cultured at 26°C. In addition, the F1 antigen in χ 10068 was synthesized and secreted on the surface of bacteria at 37°C (mammalian body temperature), not at ambient culture temperature (26°C). Immunization with χ 10068 primed antibody responses and specific T-cell responses to F1 and YpL (*Y. pestis* whole cell lysate). Oral immunization with a single dose of χ 10068 provided 70% protection against a subcutaneous (s.c.) challenge with $\sim 2.6 \times 10^5$ LD₅₀ of *Y. pestis* KIM6+ (pCD1Ap) (KIM6+Ap) and 90% protection against an intranasal (i.n.) challenge with ~ 500 LD₅₀ of KIM6+Ap in mice. Our results suggest that χ 10068 can be used as an **effective** precursor to make a safe vaccine to prevent plague in humans and to eliminate plague circulation among humans and animals.

Keywords

Yersinia pseudotuberculosis; F1; plague; vaccine

1. Introduction

Yersinia pestis, the causative agent of plague, has profoundly shaped human history by causing an estimated 200 million deaths during three major pandemics [1]. The significant

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threat of plague is due to its inherent communicability, rapid clinical course, and **high mortality if left untreated** [2]. *Y. pestis*, as a Tier 1 Select Agent, remains one of the top five bioterrorism threats [3-5]. Additionally, emergence of a *Y. pestis* strain resistant to eight antibiotics becomes a **severe** public health concern [6-8]. Therefore, prophylactic vaccination against this disease holds the brightest prospect for its long-term prevention.

Yptb PB1+ (serotype O:1b) is thought to be the direct evolutionary ancestor of *Y. pestis* [9] and a food-borne bacterial pathogen that causes infections in mammals, including humans [10]. There are several logical reasons to develop Yptb as a plague vaccine: 1) as an enteric bacterium, its use as an oral vaccine is a logical approach. 2) This bacterium causes a zoonotic disease but is much less virulent than *Y. pestis* and typically causes an enteric disease that is rarely fatal [11]. Its animal reservoirs include many mammals, such as rodents, cattle, sheep and others [12]. 3) *Y. pestis* arose from a Yptb ancestor within the last **40,000** years and the two species share high genetic similarity [9, 13, 14]. Thus, antigens known to be protective against *Y. pestis*, such as LcrV, Psn [15] and YadC [16, 17], share 96-100% identity between these two pathogens [18, 19]. 4) Pathogenic *Yersinia* species share the Yop virulon, encoded on the shared 70-kb plasmid (pCD1/pYV), which is the core of the *Yersinia* pathogenicity machinery including the T3SS. 5) Yptb has a much lower number of insertion sequences compared to *Y. pestis*, so genetically it is much more stable than the later [14]. 6) Previous studies have shown that oral immunization with attenuated Yptb strains stimulate cross-immunity to *Y. pestis* and provide partial protection against pneumonic plague in mice [11, 20, 21].

Y. pestis produces a specific capsule composed of the fraction I (F1 antigen) at mammalian temperature (37°C). The monomer of the proteinaceous capsule is 17.5-kDa [22, 23], confers resistance to phagocytosis [24], but has good immunogenic properties in *Y. pestis* [25]. African green monkeys inhaled F1-negative or F1-positive ***Y. pestis*** died at 4 to 10 days post-exposure and had lesions consistent with primary pneumonic plague [26]. F1 antigen encoded by *cafI* has been doubted as a necessary antigen, but vaccination of mice with F1 yields serum that protects mice and rats from s.c. challenge with *Y. pestis*. F1 also protects macaques against pneumonic plague by passive transfer of sera collected from F1-vaccinated rabbits [27]. Therefore, expressing ***cafI* in Yptb may augment protective immunity against *Y. pestis* strains**. However, we observed that high levels of F1 were synthesized at both 26°C and 37°C in Yptb harboring *cafI* operon expression plasmid even a pSC101 plasmid (a low copy plasmid), which seriously retards growth of bacteria at both temperatures (unpublished data). In this study, we introduced the *cafI* operon into the chromosome of Yptb mutants to eliminate the effect of high *cafI* expression on bacterial growth and demonstrated that synthesis of F1 was not in χ 10068 at ambient culture temperature (26°C), but was at 37°C. In addition, insertion of the *cafI* operon into the chromosome did not retard the growth of χ 10068 compared to wild-type Yptb cultured at 26°C. Oral immunization with a single dose of χ 10068 primed both antibody responses and specific T-cell responses to F1 and YpL (*Y. pestis* whole cell lysate) and provided **statistically significant** protection against bubonic and pneumonic plague.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and culture conditions

Table 1 listed all bacterial strains and plasmids used in this study. *E. coli* Top10 and χ 6212 used as intermediate hosts for cloning and *E. coli* χ 7213 used as a suicide plasmid donor strain were grown routinely at 37°C in LB broth [28] or on LB agar plate. Yptb PB1+ was grown in LB medium at 26°C, supplemented with chloramphenicol (50µg/ml, Cm) or arabinose (0.1%) if necessary. Tryptose blood agar (TBA) containing 5% sucrose was used for *sacB* gene-based counter selection in allelic exchange experiments. *Y. pestis* KIM6+ (pCD1Ap) [KIM6+Ap] and *Y. pestis* KIM6 (pCD1Ap) [KIM6Ap] were formed by electroporating the pCDAp plasmid into *Y. pestis* KIM6+ and *Y. pestis* KIM6, respectively (Table 1). The KIM6+Ap was used and prepared for challenge as described previously [29, 30]. The KIM6Ap was used as a vaccine standard control.

2.2. Construction of Yptb mutants

To insert the *cafIR-cafIA-cafIM-cafI* operon into *lacZ044* Yptb, the suicide plasmid pYA5184 was conjugationally transferred from *E. coli* χ 7213 [31] to χ lacZ044 Yptb PB1+. Procedures for strain construction were described in Supplementary Materials.

2.3. Animal experiments

All animal procedures were approved by Animal Care and Use Committee at the Arizona State University (Protocols #1343-R). Six-week-old, female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were acclimated for one week after arrival and deprived of food and water for 6 h before starting experiments. The procedures for animal inoculation and colonization were described in our previous report [32]. Briefly, Bacteria were harvested by centrifugation at 24°C and resuspended in 0.5 ml buffered saline with gelatin (BSG). Five mice per group were orally inoculated with approximately 10⁸ or 10⁹ CFU of bacteria in 20 µl of BSG by placing pipette tip behind the incisors of mice. Actual numbers of colony-forming units (CFU) inoculated were determined by plating serial dilutions onto LB agar.

To evaluate colonization, three mice per group were euthanized on days 3, 6 and 9 after inoculation. Spleen, liver and Peyer's patches (PPs) were collected and weighed, and BSG buffer [33] was added to a final volume of 1 ml. Samples were homogenized and plated onto MacConkey agar supplemented with 1% lactose to determine the number of viable bacteria. The detection limit was 2 CFU. For representation in graphic and statistical analysis, log₁₀ was applied to the values, and recovery of 0 CFU was reported as 1 CFU/g. All the experiments were done twice, and data were combined to calculate the LD₅₀ and colonization titers, respectively.

2.4. Determination of protective efficacy

Mice (10 mice per group) were immunized orally with 20 µl 1 × 10⁹ CFU of each strain or BSG as a negative control on day 0. Blood was obtained by mandibular vein puncture at biweekly intervals, and serum was collected individually after centrifugation for analysis of antibody response by enzyme-linked immunosorbent assay (ELISA) as described previously

[15, 34]. On day 35 after initial immunization, animals were challenged s.c. with 1×10^6 CFU of KIM6+Ap in 100 μ l PBS or lightly anesthetized with a 1:5 xylazine/ketamine mixture for i.n. challenge with 1.0×10^4 CFU of KIM6+Ap in 20 μ l PBS. All infected animals were observed over a 15-day period for recording survival.

2.5. Analysis of cellular immune responses in vitro

Spleens taken aseptically from euthanized mice (4 mice/group) were dissociated individually. The splenic cell suspensions were depleted of red blood cells (RBC) using RBC lysis buffer (Sigma) and splenocytes were washed extensively with cold PBS. T cells were purified from splenocytes by MagniSort™ Mouse T-cell Enrichment Kit according to instruction (eBiosciences, CA). Purity was routinely more than 90%, as assessed by flow cytometry using monoclonal antibodies specific for mouse CD4, CD8, and CD3 cells. Cells resuspended in RPMI 1640+Glutamax™ (Gibco) supplemented with 5% fetal bovine serum and 100 μ g/ml penicillin/streptomycin were seeded in 96 well plates (1×10^6 /well) and stimulated with either YpL (4 μ g/ml), the F1 antigen (4 μ g/ml) or PMA (20 ng/ml) plus ionomycin (1 μ g/ml) as a positive control. After 72 h, the supernatants of cell cultures were collected for cytokine analysis using multiplex assay (Bio-Rad). The cells stained with CD4-phycoerythrin (PE) (clone RM4-5), CD8-allophycocyanin (APC) (clone 53-6.7) and then intracellularly stained with IFN- γ -fluorescein isothiocyanate (FITC) (clone XMG1.2) were analyzed by Flow cytometry as mentioned in a previous report [35]. Data was collected on a Beckman Coulter FC500 and analyzed using FCS Express 4 software.

2.6. Statistical analysis

The log-rank (Mantel-Cox) test, the two-tailed t-test and One-way ANOVA were used for survival analysis, statistical analyses of spleen weight and cytokine analysis, respectively. Data was expressed as means $\pm$ SE. A *P*-value of <0.05 was considered significant.

3. RESULTS

3.1. Construction of a live attenuated Yptb strain expressing *caf1* regulated by temperature

Similar as *Y. pestis* KIM strains [36-38], no β -galactosidase activity was observed in Yptb PB1+ and disruption of *lacZ* in Yptb PB1+ did not change its virulence in mice (unpublished data). Thus, the *lacZ* location may be a good site for insertion of heterologous genes. Immunization with *Salmonella* vaccines synthesizing F1 antigen induced protective immunity against plague [39-43]. However, the virulence of *S. typhimurium* P1 containing a *caf1* expression plasmid was dramatically reduced due to its growth retardation and the disruption of its membrane caused by F1 overexpression [44, 45]. Our observations also showed that the F1 overexpression in Yptb PB1+ strain containing a *caf1* expression plasmid severely affected its growth, could not colonize in spleens and livers of mice and barely induced protective immunity against plague (data not shown). In order to overcome this problem, we inserted *caf1* operon into the *lacZ* site in the chromosome of Yptb PB1+ to construct χ 10061. Then, we added *yopK* or *yopK yopJ* mutations into χ 10061 to construct χ 10062 and χ 10068, respectively (Table 1).

We did not observe any growth retardation of either mutant strain monitored for 40 h in LB broth at 26°C, when compared to the growth of wild-type Yptb PB1+ (Figure 1A). Thus, the result indicated that chromosome insertion of the *cafI* operon or adding the single *yopK* mutation and the double *yopK* and *yopJ* mutations did not affect bacterial growth at this temperature. Similar levels of F1 synthesis were detected in χ 10061, χ 10062 and χ 10068 cultured at 37°C, but no F1 at 26°C (Figure 1B). No F1 was observed in χ 10060 (*lacZ* Yptb) as a negative control at both temperature (Figure 1B). Since significant levels of F1 were produced in χ 10061, χ 10062 and χ 10068 cultured at 37°C, we chose χ 10068 as a representative for further analysis. An immunofluorescence assay showed that F1 synthesis was observed on the surface of χ 10068 cultured at 37°C, but not at 26°C (Figure 1C). No fluorescent reactivity was observed on the surface of wild-type Yptb and χ 10060 as negative controls (data not shown). These results indicated that the F1 antigen could be successfully transported to the cell surface of Yptb as observed in *Y. pestis* [46]. Additionally, we monitored F1 synthesis by χ 10068 in vitro when χ 10068 cultures were switched from 26°C to 37°C. Results showed that F1 started to be synthesized at 1.5 h after the temperature switch (Figure 1 D).

3.2. Virulence and persistence of Yptb constructs in mice

Compared to the wild-type Yptb PB1+ (LD₅₀, 1.5×10^8 CFU) [32], 9 of 13 Swiss Webster mice survived oral administration of 1.2×10^9 CFU χ 10061, which indicated that the *cafI* operon chromosome insertion resulted in around 10-fold attenuation (**its LD₅₀, more than 1.2×10^9 CFU**). χ 10062 with additional *yopK* mutation, and χ 10068 with additional *yopK* *yopJ* mutations were much attenuated. No deaths were observed when mice were administrated orally with 1.8×10^9 CFU of χ 10062 or 1.9×10^9 CFU of χ 10068, but most of the mice showed slight weight loss and a few mice exhibited splenomegaly two weeks after inoculation. In addition, mice surviving the above-described virulence evaluations were challenged intranasally with virulent *Y. pestis* to determine which construction was worthwhile for further evaluation. Results indicated that 90% of the mice infected by χ 10068 survived i.n. challenge with 2.6×10^4 CFU of KIM6+Ap, but only 40% of the mice infected by χ 10062 survived the same challenge. Thus, χ 10068 was used for the following studies.

We evaluated dissemination of χ 10068 into Peyer's patches, spleens and livers of mice compared to the wild-type Yptb PB1+ after oral inoculation over a 9-day period. We orally infected groups of mice with 1.2×10^9 CFU of Yptb PB1+ or 1.3×10^9 CFU of χ 10068. In Peyer's patches, spleens and livers, the titers of Yptb PB1+ steadily increased at 3 and 6 days post-infection and slightly decreased at 9 days post-infection, while the titers of the χ 10068 were significantly lower than those of Yptb PB1+ at different times (Fig. 2A, B and C). In Peyer's patches, the titers of χ 10068 were almost similar at 3 and 6 days post infection, but significantly decreased at 9 days post infection (Figure 2A). In spleens and livers, the titers of χ 10068 at 3 days post infection were around 0.5 logs higher than that at 6 days post infection, whereas the titers of χ 10068 reduced much at 9 days post infection (Fig. 2B and C). At 14 days post-infection, no bacteria were detected in spleens and livers of mice infected with χ 10068, but few bacteria were detected in Peyer's patches (data not shown).

3.3. Evaluation of protective immunity

Groups of mice (20 mice per group) were orally immunized with a single dose of $\chi 10068$ (1.6×10^9 CFU), with BSG as a negative control. **One group mice** was subcutaneously immunized with 1.5×10^7 CFU of KIM6Ap (Pgm⁻) as a standard plague vaccine control that is similar to EV76, a Pgm⁻ vaccine strain developed for human use nearly a century ago [47, 48]. Results showed that oral immunization with a single dose of $\chi 10068$ provided 70% protection against an s.c. challenge with 2.6×10^6 CFU ($\sim 2.6 \times 10^5$ LD₅₀) of KIM6+Ap ($p < 0.0001$), while s.c. immunization with a single dose of KIM6Ap (Pgm⁻) provided 90% protection against the same challenge dose (Fig. 3A). The level of protection against an i.n. challenge with 5.0×10^4 CFU (~ 500 LD₅₀) of KIM6+Ap was 90% ($p < 0.0001$) in mice orally immunized with $\chi 10068$, whereas 60% protection in mice subcutaneously immunized with KIM6Ap (Pgm⁻) (Fig. 3B). None of the **BSG-immunized mice** survived the *Y. pestis* challenge by either route (Fig. 3A and B).

3.4. Serum immune responses

Measurement of total IgG responses to F1 and YpL indicated that the considerable levels of anti-F1 titers and of anti-YpL titers induced were similar between KIM6Ap (Pgm⁻) and $\chi 10068$ by week 2 and were slightly boosted by week 4 (Fig. 4A). The levels of anti-F1 titers induced by KIM6Ap (Pgm⁻) were a bit higher than those induced by $\chi 10068$ at week 4, but there were no significant differences (Fig. 4A). However, the levels of anti-YpL titers induced by KIM6Ap (Pgm⁻) were significantly higher than those induced by $\chi 10068$ at week 4 ($p < 0.05$) (Fig. 4B). No anti-F1 and anti-YpL responses were observed in the BSG immunized group (Fig. 4A and B). The levels of IgG1 and IgG2a antibodies to F1 and YpL also rapidly increased after vaccination with $\chi 10068$ at 2 weeks and boosted slightly at 4 weeks. The ratio of IgG1 to IgG2a to both F1 and YpL was near to 1 in mice immunized with $\chi 10068$ (Fig. 4C and D). The results indicated that oral immunization with $\chi 10068$ induced a Th1/Th2 balanced response to F1 and YpL.

3.5. Cellular immune response in mice orally immunized with $\chi 10068$

Studies have demonstrated that protection against pneumonic plague requires cell mediated immunity induced by INF- γ and TNF- α [49, 50]. IL-17 also contributes to cell-mediated defense against pulmonary *Y. pestis* infection [51]. Here, we determined the ability of $\chi 10068$ to induce a cellular immune response by comparing cytokine production and the populations of activated CD4⁺ and CD8⁺ T cells from splenocytes restimulated with antigens. The supernatants of these cultures were collected and analyzed by a mouse multiplex assay. Results showed that splenic cells from mice vaccinated with $\chi 10068$ produced significantly higher levels of INF- γ and IL-17 in response to restimulation with F1 antigen and YpL than mice vaccinated with BSG ($p < 0.001$) (Fig. 5 A, B, C and D). The production levels of INF- γ and IL-17 in supernatant of F1 stimulated splenocytes were a bit higher than those after YpL stimulation (Fig. 5 A, B, C and D), but there was no significant difference in IL-17 production. **However, we did not observe much TNF- α , IL-4, IL-10 and IL-12 production upon F1 and YpL stimulation or any significant difference in these cytokine levels between BSG and $\chi 10068$ -immunized groups (Figure S1).**

To further dissect what subsets of T-cell populations were responsible for F1 and YpL stimulation, outer-membrane T-cell markers and intracellular cytokine staining assay of the restimulated cells was conducted by FACS analysis. Analysis results demonstrated that strong IFN- γ staining in cells stimulated by both F1 and YpL antigens was observed in both CD4⁺ and CD8⁺ T cells (**Fig. 5 E & F, and Figure S2**), whereas significantly lower number of cells from control mice stained with IFN- γ . The results demonstrated that oral immunization with χ 10068 clearly induced cellular immune responses specific to F1 and *Y. pestis*.

4. DISCUSSION

Plague, a zoonotic disease primarily affecting rodents and their associated fleas, has an obligate life cycle alternating between growth in mammals and fleas [52], making it difficult to control. A viable strategy targeting wild rodent populations against plague, especially those living in close contact with humans would directly address the source of *Y. pestis* and prevent its spread to humans. As an enteric pathogen, Yptb has a broad host range in mammals (such as rodents, dogs, cats, cattle, rabbits, deer and humans) [10, 12, 53]. Modified Yptb as an oral vaccine delivered by baits to wild animals might be a potential means to control or eradicate the transmission of sylvatic plague in its natural rodent hosts to humans.

So far, little research has been conducted by using Yptb as a vaccine against plague. Wake et al. first reported that subcutaneous vaccination with a live virulent strain of Yptb (10^2 CFU) provided 50% protection of mice (dd/Y strain, outbred mice) against s.c. challenge with 5.2×10^5 *Y. pestis* Yreka strain ($\sim 10^5$ LD₅₀) [54]. Intravenous injection of 10^4 CFU Yptb strain 2637b (a pYV-cured strain) provided a 50% protection against bubonic plague [55]. Later on, Taylor et al. demonstrated that a single dose of the Yptb IP32953 *dam* pYV-mutant (10^9 CFU) delivered orally induced efficient protection (80% survival of mice) against s.c. challenge with 100 CFU of the *Y. pestis* GB strain [20]. Yptb IP32680 (with absence of the high pathogenicity island, the super antigens YPM, and the type IV pilus) was avirulent in mice upon intragastric or subcutaneous inoculation. Mice vaccinated orally once or twice at a 30-day interval with IP32680 were subcutaneously challenged with 1,500 CFU (150 LD₅₀) of *Y. pestis* strain CO92 at 30 days after the last immunization. 75% and 88% of the above immunized mice survived the s.c. challenge, respectively. However, only 30% of mice immunized orally twice with IP32680 survived intranasal challenge with around $\sim 8,000$ CFU of *Y. pestis* CO92 [11]. Our results showed that oral immunization with a single dose of χ 10068 provided 70% protection against an s.c. challenge with very high dose of KIM6+Ap (2.6×10^6 CFU, $\sim 2.6 \times 10^5$ LD₅₀) (Fig. 3A). The level of protection against i.n. challenge with a ~ 500 LD₅₀ (5.0×10^4 CFU) of KIM6+Ap was 90% protection in mice immunized with χ 10068 (Fig. 3B). Although the challenge strains used by the two research groups were different, the LD₅₀s of KIM6+Ap and *Y. pestis* CO92 are similar [56, 57]. Thus, χ 10068 definitely is a very promising candidate for further modification.

Humoral and cellular immunity are important for protection against plague [58, 59]. Previous studies also have confirmed the role of cytokines IFN- γ , TNF- α , and IL-17A in plague protection [60-64]. Our results indicated that immunization with χ 10068 induced

considerable antibody titers to F1 and YpL and a Th1/Th2 balanced response to F1 and YpL (Fig. 4C and D). However, no significant secretory IgA (sIgA) to F1 and YpL in vaginal washes of mice orally vaccinated with χ 10068 was observed, and even IgA deficient mice immunized with χ 10068 had same protection against pneumonic plague as wild-type mice (unpublished data). Splenic cells from mice vaccinated with χ 10068 produced significant levels of IFN- γ and IL-17 upon F1 and YpL restimulation (Fig. 5). In Figure 5, levels of IFN- γ , and IL-17 production upon YpL restimulation were lower than for F1 stimulation. Since YpL prepared from a lysate of KIM6+Ap cultured at 37°C [56] contained massive tetra-acylated lipid A [29, 65-68], which might inhibit the induction of proinflammatory cytokines. T-cell-mediated responses are important for the clearance of primary infections, as well as for conferring long-term immunity to plague [49, 50]. Immunization with live attenuated Yptb strains was able to stimulate antigen-specific T-cell responses [69, 70]. Our results also demonstrated that χ 10068 immunization induced significant CD4+ and CD8+ T-cell responses to F1 and *Y. pestis* (Figure 5 E & F). Therefore, considerable levels of antibody responses, IFN- γ , IL-17 and T-cell responses upon *Y. pestis* infection induced in mice immunized with χ 10068 do prevent *Y. pestis* infection.

The *lcrV* polymorphisms that maintain the functional attributes of the type III secretion pathway may result in variants that escape plague protective immunity [71, 72]. Antibodies raised against LcrV_{D27} were unable to block the type III injection of *Y. enterocolitica* strains, but 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 [73]. Regarding concerns about the *lcrV* polymorphisms and F1⁻ *Y. pestis* strain, we will test our live attenuated Yptb vaccines against F1 negative *Y. pestis* strain or other *Y. pestis* expressing different *lcrV* in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference

1. Wagner DM, Klunk J, Harbeck M, Devault A, Waglechner N, Sahl JW, et al. *Yersinia pestis* and the Plague of Justinian 541-543 AD: a genomic analysis. *The Lancet infectious diseases*. 2014; 14:319–26. [PubMed: 24480148]
2. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, et al. Plague: Past, Present, and Future. *PLoS medicine*. 2008; 5:e3. [PubMed: 18198939]
3. Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Plague as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *JAMA: The Journal Of The American Medical Association*. 2000; 283:2281–90.
4. Perry RD, Fetherston JD. *Yersinia pestis*--etiologic agent of plague. *Clin Microbiol Rev*. 1997; 10:35–66. [PubMed: 8993858]
5. Prentice MB, Rahalison L. Plague. *Lancet*. 2007; 369:1196–207. [PubMed: 17416264]

6. Guiyoule A, Rasoamanana B, Buchrieser C, Michel P, Chanteau S, Carniel E. Recent emergence of new variants of *Yersinia pestis* in Madagascar. J Clin Microbiol. 1997; 35:2826–33. [PubMed: 9350742]
7. Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, et al. Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of *Yersinia pestis*. Emerg Infect Dis. 2001; 7:43–8. [PubMed: 11266293]
8. Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, et al. Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. N Engl J Med. 1997; 337:677–80. [PubMed: 9278464]
9. Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A. 1999; 96:14043–8. [PubMed: 10570195]
10. Laukkanen-Ninios R, Didelot X, Jolley KA, Morelli G, Sangal V, Kristo P, et al. Population structure of the *Yersinia pseudotuberculosis* complex according to multilocus sequence typing. Environmental microbiology. 2011; 13:3114–27. [PubMed: 21951486]
11. Blisnick T, Ave P, Huerre M, Carniel E, Demeure CE. Oral vaccination against bubonic plague using a live avirulent *Yersinia pseudotuberculosis* strain. Infect Immun. 2008; 76:3808–16. [PubMed: 18505804]
12. Howard IS, Ingram JN, Wolpert DM. Context-dependent partitioning of motor learning in bimanual movements. J Neurophysiol. 2010; 104:2082–91. [PubMed: 20685927]
13. Achtman M. Age, descent and genetic diversity within *Yersinia pestis*. In: Carniel E.; Hinnebusch, BJ., editors. *Yersinia: Molecular and Cellular Biology*. Horizon Bioscience; Norwich, UK: 2004. p. 432
14. Chain PS, Carniel E, Larimer FW, Lamerdin J, Stoutland PO, Regala WM, et al. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A. 2004; 101:13826–31. [PubMed: 15358858]
15. Branger CG, Sun W, Torres-Escobar A, Perry R, Roland KL, Fetherston J, et al. 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. Vaccine. 2010; 29:274–82. [PubMed: 20979987]
16. Murphy BS, Wulff CR, Garvy BA, Straley SC. *Yersinia pestis* YadC: a novel vaccine candidate against plague. Adv Exp Med Biol. 2007; 603:400–14. [PubMed: 17966436]
17. Sun W, Olinzock J, Wang S, Sanapala S, Curtiss R 3rd. Evaluation of YadC protein delivered by live attenuated *Salmonella* as a vaccine against plague. Pathog Dis. 2013
18. Buchrieser C, Rusniok C, Frangeul L, Couve E, Billault A, Kunst F, et al. The 102-kilobase pgm locus of *Yersinia pestis*: sequence analysis and comparison of selected regions among different *Yersinia pestis* and *Yersinia pseudotuberculosis* strains. Infect Immun. 1999; 67:4851–61. [PubMed: 10456941]
19. Forman S, Wulff CR, Myers-Morales T, Cowan C, Perry RD, Straley SC. *yadBC* of *Yersinia pestis*, a new virulence determinant for bubonic plague. Infect Immun. 2008; 76:578–87. [PubMed: 18025093]
20. Taylor VL, Titball RW, Oyston PC. Oral immunization with a *dam* mutant of *Yersinia pseudotuberculosis* protects against plague. Microbiology. 2005; 151:1919–26. [PubMed: 15941999]
21. Okan NA, Mena P, Benach JL, Bliska JB, Karzai AW. The *smpB-ssrA* mutant of *Yersinia pestis* functions as a live attenuated vaccine to protect mice against pulmonary plague infection. Infect Immun. 2010; 78:1284–93. [PubMed: 20065026]
22. Galyov EE, Karlishev AV, Chernovskaya TV, Dolgikh DA, Smirnov O, Volkovoy KI, et al. Expression of the envelope antigen F1 of *Yersinia pestis* is mediated by the product of *cafIM* gene having homology with the chaperone protein PapD of *Escherichia coli*. FEBS Lett. 1991; 286:79–82. [PubMed: 1677900]
23. Karlyshev AV, Galyov EE, Smirnov O, Guzayev AP, Abramov VM, Zav'yalov VP. A new gene of the *f1* operon of *Y. pestis* involved in the capsule biogenesis. FEBS Lett. 1992; 297:77–80. [PubMed: 1551441]

24. Rodrigues CG, Carneiro CM, Barbosa CT, Nogueira RA. Antigen F1 from *Yersinia pestis* forms aqueous channels in lipid bilayer membranes. *Braz J Med Biol Res.* 1992; 25:75–9. [PubMed: 1284806]
25. Meyer KF, Hightower JA, McCrumb FR. Plague immunization. VI. Vaccination with the fraction I antigen of *Yersinia pestis*. *The Journal of infectious diseases.* 1974; 129(Suppl):S41–5. [PubMed: 4825248]
26. Davis KJ, Fritz DL, Pitt ML, Welkos SL, Worsham PL, Friedlander AM. Pathology of experimental pneumonic plague produced by fraction I-positive and fraction I-negative *Yersinia pestis* in African green monkeys (*Cercopithecus aethiops*). *Arch Pathol Lab Med.* 1996; 120:156–63. [PubMed: 8712895]
27. Quenee LE, Cornelius CA, Ciletti NA, Elli D, Schneewind O. *Yersinia pestis* *cafI* variants and the limits of plague vaccine protection. *Infect Immun.* 2008; 76:2025–36. [PubMed: 18347051]
28. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol.* 1951; 62:293–300. [PubMed: 14888646]
29. Sun W, Six D, Kuang XY, Roland KL, Raetz CRH, Curtiss R III. A live attenuated strain of *Yersinia pestis* KIM as a vaccine against plague. *Vaccine.* 2011; 29:2986–98. [PubMed: 21320544]
30. Sun W, Roland KL, Kuang X, Branger CG, Curtiss R III. *Yersinia pestis* with regulated delayed attenuation as a vaccine candidate to induce protective immunity against plague. *Infect Immun.* 2010; 78:1304–13. [PubMed: 20086087]
31. Wang S, Li Y, Scarpellini G, Kong W, Shi H, Baek CH, et al. *Salmonella* vaccine vectors displaying delayed antigen synthesis in vivo to enhance immunogenicity. *Infection and immunity.* 2010; 78:3969–80. [PubMed: 20605977]
32. Sun W, Sanapala S, Henderson JC, Sam S, Olinzock J, Trent MS, et al. LcrV delivered via type III secretion system of live attenuated *Yersinia pseudotuberculosis* enhances immunogenicity against pneumonic plague. *Infection and immunity.* 2014; 82:4390–404. [PubMed: 25114109]
33. Wang S, Li Y, Shi H, Sun W, Roland KL, Curtiss R 3rd. Comparison of a regulated delayed antigen synthesis system with in vivo-inducible promoters for antigen delivery by live attenuated *Salmonella* vaccines. *Infection and immunity.* 2011; 79:937–49. [PubMed: 21134969]
34. Kang HY, Srinivasan J, Curtiss R III. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar *typhimurium* vaccine. *Infection and immunity.* 2002; 70:1739–49. [PubMed: 11895935]
35. Wang S, Goguen JD, Li F, Lu S. Involvement of CD8+ T cell-mediated immune responses in LcrV DNA vaccine induced protection against lethal *Yersinia pestis* challenge. *Vaccine.* 2011; 29:6802–9. [PubMed: 21199697]
36. Staggs TM, Perry RD. Identification and cloning of a *fur* regulatory gene in *Yersinia pestis*. *Journal of bacteriology.* 1991; 173:417–25. [PubMed: 1898928]
37. Geoffroy VA, Fetherston JD, Perry RD. *Yersinia pestis* YbtU and YbtT are involved in synthesis of the siderophore yersiniabactin but have different effects on regulation. *Infect Immun.* 2000; 68:4452–61. [PubMed: 10899842]
38. Bobrov AG, Perry RD. *Yersinia pestis* *lacZ* expresses a beta-galactosidase with low enzymatic activity. *FEMS Microbiol Lett.* 2006; 255:43–51. [PubMed: 16436060]
39. Galen JE, Wang JY, Carrasco JA, Lloyd SA, Mellado-Sanchez G, Diaz-McNair J, et al. A bivalent typhoid live vector vaccine expressing both chromosome-and plasmid-encoded *Yersinia pestis* antigens fully protects against murine lethal pulmonary plague infection. *Infection and immunity.* 2015; 83:161–72. [PubMed: 25332120]
40. Yang X, Hinnebusch BJ, Trunkle T, Bosio CM, Suo Z, Tighe M, et al. Oral vaccination with *salmonella* simultaneously expressing *Yersinia pestis* F1 and V antigens protects against bubonic and pneumonic plague. *J Immunol.* 2007; 178:1059–67. [PubMed: 17202369]
41. Morton M, Garmory HS, Perkins SD, O'Dowd AM, Griffin KF, Turner AK, et al. A *Salmonella enterica* serovar Typhi vaccine expressing *Yersinia pestis* F1 antigen on its surface provides protection against plague in mice. *Vaccine.* 2004; 22:2524–32. [PubMed: 15193377]

42. Titball RW, Howells AM, Oyston PC, Williamson ED. Expression of the *Yersinia pestis* capsular antigen (F1 antigen) on the surface of an *aroA* mutant of *Salmonella typhimurium* induces high levels of protection against plague. *Infect Immun*. 1997; 65:1926–30. [PubMed: 9125581]
43. Oyston PC, Williamson ED, Leary SE, Eley SM, Griffin KF, Titball RW. Immunization with live recombinant *Salmonella typhimurium aroA* producing F1 antigen protects against plague. *Infect Immun*. 1995; 63:563–8. [PubMed: 7822022]
44. Pascual DW, Suo Z, Cao L, Avci R, Yang X. Attenuating gene expression (AGE) for vaccine development. *Virulence*. 2013; 4:384–90. [PubMed: 23652809]
45. Cao L, Lim T, Jun S, Thornburg T, Avci R, Yang X. Vulnerabilities in *Yersinia pestis* *caf* operon are unveiled by a *Salmonella* vector. *PLoS ONE*. 2012; 7:e36283. [PubMed: 22558420]
46. Vorontsov ED, Dubichev AG, Serdobintsev LN, Naumov AV. Association-dissociation processes and supermolecular organisation of the capsule antigen (protein F1) of *Yersinia pestis*. *Biomed Sci*. 1990; 1:391–6. [PubMed: 2133058]
47. Brubaker RR. The Vwa+ virulence factor of yersiniae: the molecular basis of the attendant nutritional requirement for Ca²⁺. *Rev Infect Dis*. 1983; 5(Suppl 4):S748–58. [PubMed: 6195719]
48. Girard G. Immunity in plague. Acquisitions supplied by 30 years of work on the “*Pasteurella pestis* EV” (Girard and Robic) strain. *Biol Med (Paris)*. 1963; 52:631–731. [PubMed: 14095162]
49. Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, et al. Cell-mediated protection against pulmonary *Yersinia pestis* infection. *Infect Immun*. 2005; 73:7304–10. [PubMed: 16239527]
50. Parent MA, Wilhelm LB, Kummer LW, Szaba FM, Mullarky IK, Smiley ST. Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary *Yersinia pestis* infection. *Infect Immun*. 2006; 74:3381–6. [PubMed: 16714568]
51. Lin JS, Kummer LW, Szaba FM, Smiley ST. IL-17 Contributes to Cell-Mediated Defense against Pulmonary *Yersinia pestis* Infection. *J Immunol*. 2011
52. Gratz, NG. Rodent reservoirs and flea vectors of natural foci of plague.. In: Dennis, DT.; Gage, KL.; Gratz, NG.; Poland, JD.; Thikhomirov, E., editors. *Plague manual: epidemiology, distribution, surveillance and control*. World Health Organization; Geneva: 1999. p. 63-96.
53. Gaydos JK, Zabek E, Raverty S. *Yersinia pseudotuberculosis* septicemia in a beaver from Washington State. *J Wildl Dis*. 2009; 45:1182–6. [PubMed: 19901394]
54. Wake A, Morita H, Wake M. Mechanisms of long and short term immunity to plague. *Immunology*. 1978; 34:1045–52. [PubMed: 680791]
55. Simonet M, Berche P, Mazigh D, Veron M. Protection against *Yersinia* infection induced by non-virulence-plasmid-encoded antigens. *J Med Microbiol*. 1985; 20:225–31. [PubMed: 3900406]
56. Sun W, Roland KL, Branger CG, Kuang X, Curtiss R 3rd. The role of *relA* and *spoT* in *Yersinia pestis* KIM5+ pathogenicity. *PLoS One*. 2009; 4:e6720. [PubMed: 19701461]
57. Lathem WW, Crosby SD, Miller VL, Goldman WE. Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. *Proc Natl Acad Sci U S A*. 2005; 102:17786–91. [PubMed: 16306265]
58. Li B, Du C, Zhou L, Bi Y, Wang X, Wen L, et al. Humoral and cellular immune responses to *Yersinia pestis* infection in long-term recovered plague patients. *Clinical and vaccine immunology : CVI*. 2012; 19:228–34. [PubMed: 22190397]
59. Li B, Yang R. Interaction between *Yersinia pestis* and the host immune system. *Infect Immun*. 2008; 76:1804–11. [PubMed: 18250178]
60. Derbise A, Cerda Marin A, Ave P, Blisnick T, Huerre M, Carniel E, et al. An encapsulated *Yersinia pseudotuberculosis* is a highly efficient vaccine against pneumonic plague. *Plos Neglect Trop D*. 2012; 6:e1528.
61. Lukaszewski RA, Kenny DJ, Taylor R, Rees DG, Hartley MG, Oyston PC. Pathogenesis of *Yersinia pestis* infection in BALB/c mice: effects on host macrophages and neutrophils. *Infect Immun*. 2005; 73:7142–50. [PubMed: 16239508]
62. Pujol C, Grabenstein JP, Perry RD, Bliska JB. Replication of *Yersinia pestis* in interferon gamma-activated macrophages requires *ripA*, a gene encoded in the pigmentation locus. *Proc Natl Acad Sci U S A*. 2005; 102:12909–14. [PubMed: 16120681]

63. Nakajima R, Brubaker RR. Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. *Infect Immun*. 1993; 61:23–31. [PubMed: 8418045]
64. Elvin SJ, Williamson ED. Stat 4 but not Stat 6 mediated immune mechanisms are essential in protection against plague. *Microb Pathog*. 2004; 37:177–84. [PubMed: 15458778]
65. Kawahara K, Tsukano H, Watanabe H, Lindner B, Matsuura M. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect Immun*. 2002; 70:4092–8. [PubMed: 12117916]
66. Rebeil R, Ernst RK, Gowen BB, Miller SI, Hinnebusch BJ. Variation in lipid A structure in the pathogenic yersiniae. *Mol Microbiol*. 2004; 52:1363–73. [PubMed: 15165239]
67. Knirel YA, Lindner B, Vinogradov EV, Kocharova NA, Senchenkova SN, Shaikhutdinova RZ, et al. Temperature-dependent variations and intraspecies diversity of the structure of the lipopolysaccharide of *Yersinia pestis*. *Biochemistry*. 2005; 44:1731–43. [PubMed: 15683257]
68. Sun W, Six DA, Reynolds CM, Chung HS, Raetz CR, Curtiss R 3rd. Pathogenicity of *Yersinia pestis* synthesis of 1-dephosphorylated lipid A. *Infection and immunity*. 2013; 81:1172–85. [PubMed: 23357387]
69. Russmann H, Gerdemann U, Igwe EI, Panthel K, Heesemann J, Garbom S, et al. Attenuated *Yersinia pseudotuberculosis* carrier vaccine for simultaneous antigen-specific CD4 and CD8 T-cell induction. *Infect Immun*. 2003; 71:3463–72. [PubMed: 12761131]
70. Wiedig CA, Kramer U, Garbom S, Wolf-Watz H, Autenrieth IB. Induction of CD8+ T cell responses by *Yersinia* vaccine carrier strains. *Vaccine*. 2005; 23:4984–98. [PubMed: 15985316]
71. Motin VL, Nakajima R, Smirnov GB, Brubaker RR. Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. *Infection and immunity*. 1994; 62:4192–201. [PubMed: 7927675]
72. Sun W, Curtiss Rr. Amino acid substitutions in LcrV at putative sites of interaction with toll-like receptor 2 do not affect the virulence of *Yersinia pestis*. *Microb Pathogenesis*. 2012; 53:198–206.
73. Miller NC, Quenee LE, Elli D, Ciletti NA, Schneewind O. Polymorphisms in the lcrV Gene of *Yersinia enterocolitica* and Their Effect on Plague Protective Immunity. *Infection and Immunity*. 2012; 80:1572–82. [PubMed: 22252870]
74. Gong S, Bearden SW, Geoffroy VA, Fetherston JD, Perry RD. Characterization of the *Yersinia pestis* Yfu ABC inorganic iron transport system. *Infect Immun*. 2001; 69:2829–37. [PubMed: 11292695]
75. Goguen JD, Yother J, Straley SC. Genetic analysis of the low calcium response in *Yersinia pestis* mu d1(Ap lac) insertion mutants. *J Bacteriol*. 1984; 160:842–8. [PubMed: 6094509]
76. Kang HY, Dozois CM, Tinge SA, Lee TH, Curtiss R 3rd. Transduction-mediated transfer of unmarked deletion and point mutations through use of counterselectable suicide vectors. *Journal of bacteriology*. 2002; 184:307–12. [PubMed: 11741872]

Highlights

A new genetically modified *Y. pseudotuberculosis* construction, χ 10068, synthesizes F1 antigen as *Y. pestis* does. Immunization with χ 10068 primed potent antibody responses and specific T-cell responses to F1 and *Y. pestis*. Oral immunization with a single dose of χ 10068 provided 70% protection against a subcutaneous challenge with $\sim 2.6 \times 10^5$ LD₅₀ of *Y. pestis* KIM6+ (pCD1Ap) and 90% protection against an intranasal challenge with ~ 500 LD₅₀ of KIM6+Ap in mice. χ 10068 definitely is a very promising candidate for further modification.

Figure 1.

Yptb growth and F1 synthesis in different Yptb constructions. (A) The growth curve of wild-type Yptb, χ 10061(*lacZ044::caf1R-caf1M-caf1A-caf1*), χ 10062 [*lacZ044::caf1R-caf1M-caf1A-caf1* pYV- ω 1 (*yopK108*)] and χ 10068 [*lacZ044::caf1R-caf1M-caf1A-caf1* pYV- ω 2 (*yopJ315 yopK108*)] in LB medium at 26°C; (B) F1 synthesis was determined by western blotting. The strains (from left to right): χ 10060 (*lacZ044*), χ 10061, χ 10062 and χ 10068 cultured at 26°C and 37°C; (C) Immunofluorescence for assaying F1 synthesis on the surface of χ 10068 cultured at 26°C and 37°C. Bacteria were incubated with primary antibody (anti-F1 monoclonal antibody) for 1 h, washed by PBS three times and then incubated with anti-mouse IgG labeled with fluorescein isothiocyanate (FITC). FITC-labeled bacteria were observed under fluorescent microscopy at 492 nm; (D) Determination of F1 synthesis in χ 10068. The χ 10068 strain was cultured at 26°C for overnight and switched to 37°C at different times.

Figure 2.

Kinetics of bacterial burden in mice infected orally with Yptb PB1+ (●), χ 10068 (▼).

Groups of mice were inoculated orally with 1.2×10^9 CFU of wild-type *Y.*

pseudotuberculosis PB1+, 1.3×10^9 CFU of χ 10068. CFU per organ in Peyer's patches (A), spleens (B) and livers (C) from 3 mice per group were determined at various times (3, 6 and 9 days). Error bars represent standard deviations. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

The experiment was performed twice and data were combined.

Figure 3.

Mouse survival after KIM6+Ap challenge. Swiss Webster mice (10 per group) orally vaccinated with a dose of 1.6×10^9 CFU of $\chi 10068$, BSG as negative control and subcutaneously vaccinated with 1.5×10^9 CFU of KIM6Ap (Pgm⁻) as a positive control. At 35 days after initial immunization, mice were challenged with $\sim 2.6 \times 10^5$ LD₅₀ (2.6×10^6 CFU) of KIM6+Ap via the s.c. route (A) and ~ 500 LD₅₀ (5.0×10^4 CFU) of KIM6+Ap via the i.n. route (B). Survival of immunized mice was significantly better than for PBS controls in all experiments. *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$. All experiments were performed twice with similar outcomes and the results were pooled for presentation. The LD₅₀s of KIM6+Ap were less 10 CFU by s.c. challenge and 100 CFU by i.n. challenge, respectively [29].

Figure 4.

Antibody responses in mice immunized with BSG and $\chi 10068$. (A) Total IgG response to F1 antigen at weeks 2 and 4 after initial immunization; (B) Total IgG response to YpL at weeks 2 and 4 after initial immunization; (C) IgG1 and IgG2a antibody levels to F1 in sera of mice orally immunized with $\chi 10068$ at weeks 2 and 4 after initial immunization; (D) IgG1 and IgG2a antibody levels to YpL in sera of mice orally immunized with $\chi 10068$ at weeks 2 and 4 after initial immunization. *: $P < 0.05$, ns: no significant difference. Significant difference between the level of IgG of $\chi 10068$ vs BSG ($P < 0.0001$).

Figure 5.

Analysis of production of cytokines and antigen-specific T cells in vitro. Splenocytes isolated from Swiss Webster mice (4 mice per group) orally vaccinated with a single dose of $\chi 10068$ (1.1×10^9 CFU) or BSG at 28 days after initial immunization were stimulated in vitro with 4 mg/ml of either a purified F1 or YpL. The PMA (20 ng/ml) plus ionomycin (1 μ g/ml) served as a positive control and RPMI 1640 media used as a negative control. Antigen-specific IFN- γ and IL-17 cytokines in the culture supernatants produced from splenic cells after 3 days of stimulation were measured by Bioplex assays. The cells stained

with CD4-phycoerythrin (PE) (clone RM4-5), CD8-allophycocyanin (APC) (clone 53-6.7) and then intracellularly stained with IFN- γ -fluorescein isothiocyanate (FITC) (clone XMG1.2) were analyzed by flow cytometry. (A) Production of IFN- γ after F1 stimulation; (B) Production of IFN- γ after YpL stimulation; (C) Production of IL-17 after F1 stimulation; and (D) Production of IL-17 after YpL stimulation. (E) Portion of CD4+ IFN- γ + T cells; (F) Portion of CD8+ IFN- γ + T cells; Data was expressed as mean $\pm$ SD. 4 mice for each experiment (results from two experiments were pooled together). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Table 1

Strains and plasmids used in this study

Strain or Plasmid	Genotype or relevant characteristics	Source
Strains		
<i>E. coli</i> Top10	F ⁻ <i>mcrA</i> (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> M15 <i>lacX74 recA1 araD139</i> (<i>ara-leu</i>)-7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Lab collection
<i>E. coli</i> χ 6212	F ⁻ λ^- ϕ 80 (<i>lacZYA-argF</i>) <i>endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 asdA4</i>	[34]
<i>E. coli</i> χ 7213	<i>thi-1 thr-1 leuB6 fluA21 lacY1 glnV44 asdA4 recA1 RP4 2-Tc::Mu [λpir]; Km^r</i>	[31]
<i>Y. pseudotuberculosis</i> PB1+	Serotype O:1B	Dr. Robert Perry
χ 10060	<i>lacZ044</i>	<i>Y. pseudotuberculosis</i> PB1+
χ 10061	<i>lacZ044::cafIR-cafIM-cafIA-cafI</i>	χ 10060
χ 10062	<i>lacZ044::cafIR-cafIM-cafIA-cafI pYV-ϕ1</i> (<i>yopK108</i>)	χ 10061
χ 10068	<i>lacZ044::cafIR-cafIM-cafIA-cafI pYV-ϕ2</i> (<i>yopJ315 yopK108</i>)	χ 10062
<i>Y. pestis</i> KIM6+	Pgm ⁺ , pMT1, pPCP1, no pCD1	[74]
<i>Y. pestis</i> KIM6	Pgm ⁻ , pMT1, pPCP1, no pCD1	[75]
<i>Y. pestis</i> KIM6+(pCD1Ap) (KIM6+Ap)	Pgm ⁺ , pMT1, pPCP1, the pCD1Ap was electoporated into <i>Y. pestis</i> KIM6+	[74]
<i>Y. pestis</i> KIM6(pCD1Ap) (KIM6AP)	Pgm ⁻ , pMT1, pPCP1, the pCD1Ap was electoporated into <i>Y. pestis</i> KIM6+	[75]
Plasmids		
pRE112	Suicide vector, Cm ^r , <i>mob</i> ⁻ (RP4)R6K <i>ori</i> , <i>sacB</i>	[76]
pYA3342	Asd ⁺ , pBR plasmid	[34]
pYA4454	Remove P _{lac} promoter from pWSK129 plasmid	
pYA5182	<i>lacZ</i> of Yptb fragment into <i>KpnI</i> and <i>XmaI</i> sites of pYA4454	This study
pYA5183	The <i>cafI</i> operon amplified from <i>Y. pestis</i> was cloned into <i>PstI</i> and <i>SacI</i> sites of pYA5182	This study
pYA5184	The fragment of <i>lacZ::cafIR-cafIM-cafIA-cafI</i> was cloned into <i>KpnI</i> and <i>XmaI</i> sites of pRE112	This study
pYA5323	The flanking regions of <i>yopK</i> of Yptb into <i>XmaI</i> and <i>KpnI</i> sites of pRE112	This study
pYA5324	The flanking regions of <i>yopJ</i> of Yptb into <i>XmaI</i> and <i>KpnI</i> sites of pRE112	This study
pYA5338	The <i>cafIM-cafIA-cafI</i> -6xHis was cloned under P _{lac} promoter of pYA3342	This study