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LcrV Delivered via Type III Secretion System of Live Attenuated *Yersinia pseudotuberculosis* Enhances Immunogenicity against Pneumonic Plague

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Here, we constructed a *Yersinia pseudotuberculosis* mutant strain with arabinose-dependent regulated and delayed shutoff of *crp* expression (*araC* P_{BAD} *crp*) and replacement of the *msbB* gene with the *Escherichia coli msbB* gene to attenuate it. Then, we inserted the *asd* mutation into this construction to form χ10057 [Δ*asd-206* Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*] for use with a balanced-lethal Asd-positive (Asd^+) plasmid to facilitate antigen synthesis. A hybrid protein composed of YopE (amino acids [aa]1 to 138) fused with full-length LcrV ($YopE_{Nt138}$-LcrV) was synthesized in χ10057 harboring an Asd^+ plasmid (pYA5199, *yopE*$_{Nt138}$-*lcrV*) and could be secreted through a type III secretion system (T3SS) *in vitro* and *in vivo*. Animal studies indicated that mice orally immunized with χ10057(pYA5199) developed titers of IgG response to whole-cell lysates of *Y. pestis* (YpL) and subunit LcrV similar to those seen with χ10057(pYA3332) (χ10057 plus an empty plasmid). However, only immunization of mice with χ10057(pYA5199) resulted in a significant secretory IgA response to LcrV. χ10057(pYA5199) induced a higher level of protection (80% survival) against intranasal (i.n.) challenge with ~240 median lethal doses (LD_{50}) (2.4×10^4 CFU) of *Y. pestis* KIM6+(pCD1Ap) than χ10057(pYA3332) (40% survival). Splenocytes from mice vaccinated with χ10057(pYA5199) produced significant levels of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-17 (IL-17) after restimulation with LcrV and YpL antigens. Our results suggest that it is possible to use an attenuated *Y. pseudotuberculosis* strain delivering the LcrV antigen via the T3SS as a potential vaccine candidate against pneumonic plague.

Yersinia pestis, the etiologic agent causing plague, has been responsible for high mortality in several epidemics throughout human history and remains a current threat as a potential biological-warfare agent (1, 2). Currently, about 2,000 global cases of plague are reported to the World Health Organization each year (3). Most of these cases are of the bubonic form, usually a consequence of the transmission of bacteria to humans via bites from fleas that had previously fed on infected rodents, while contact with domestic cats that had been exposed to *Y. pestis* is another important transmission mode because of the higher-than-average incidence of pneumonic plague that occurs in these cases (1). Additionally, there has been an emergence of a *Y. pestis* strain resistant to eight antibiotics, which is a great public health concern (4–6). Therefore, prophylactic vaccination against this disease holds the brightest prospect for its control in the long term, but no safe and efficient vaccines against plague are currently available.

The live EV76 vaccine is an apparent *pgm* mutant that has been used in some countries in the past (7). However, a concern is that the EV76 vaccine strain can cause disease in primates, thus raising questions about its suitability as a human vaccine (8). Wang et al. summarized the latest progress in live attenuated *Y. pestis* vaccine development (9). In addition, live bacteria, such as *Salmonella* species, *Escherichia coli*, or *Lactococcus lactis*, or live viruses, such as replication-deficient adenovirus, vesicular stomatitis virus, vaccinia virus, or raccoon poxvirus, have been used as vectors for the effective delivery of F1 and/or V antigen to induce protective immunity to plague (10, 11). Recently, the main focus of plague vaccine research has been to develop subunit vaccines, in particular, those targeting LcrV and F1 antigens, which were found to efficiently protect rodent and cynomolgus macaque against bubonic and pneumonic plague and are well tolerated in humans (12–20). However, the subunit vaccine had insufficient and highly variable protection against plague in African green monkeys (20, 21). Additionally, the usefulness of F1 as a protective antigen is not clear, since $F1^-$ strains can cause plague (22). Therefore, vaccines composed of a limited number of antigens (F1 and/or LcrV) may not be able to protect against F1-negative strains (22) or strains harboring LcrV variants (23). Live *Yersinia* vaccines offer several advantages over recombinant vaccines. Their high antigenic complexity guarantees a response to a broad range of antigenic targets. Additionally, they are often less expensive to manufacture than subunit vaccines (10). Thus, plague vaccines based on live attenuated *Yersinia* spp. provide the theoretical advantage of simultaneously priming immunity responses to many antigens, thereby reducing the likelihood of antigen circumvention by clever terrorists (24).

Y. pseudotuberculosis is thought to be the direct evolutionary ancestor of *Y. pestis* (25–27). The two species diverged from one another 2,600 to 28,000 years ago (27–29). But *Y. pseudotuberculosis* is much less virulent and typically causes enteric disease in humans and animals. Its infections are self-limiting with a low

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case fatality rate, and its lifestyle as an enteric pathogen will facilitate its use as an oral vaccine. With the exception of two additional plasmids (pPCP1 and pMT1) carried by *Y. pestis*, the two species share >95% genetic identity and a common virulence plasmid with a conserved colinear backbone (30). BLAST analysis of several major *Y. pestis* antigens showed that LcrV shares 96% amino acid identity between the two species and that Psn and YadC, two additional antigens shown to be protective against *Y. pestis* challenge (31–33), share 100% homology (34) and >97% homology (35), respectively. Furthermore, *Y. pseudotuberculosis* has a much lower number of insertion sequence (IS) copies than *Y. pestis* and so is genetically much more stable than the latter (30).

Oral immunization with attenuated *Y. pseudotuberculosis* strains provides partial protection against pulmonary challenge with *Y. pestis* (36–38). Recently, Derbise et al. reported that a single oral immunization of the encapsulated *Y. pseudotuberculosis* V674pF1 strain offered great protection against a lethal pneumonic plague challenge (39). Therefore, these data demonstrated the feasibility of using modified *Y. pseudotuberculosis* strains to control plague transmission and induction of mortality.

Previous studies suggest that *Y. pseudotuberculosis* is a promising candidate for an oral live carrier vaccine, capable of stimulating antigen specific $CD8^+$ T-cell responses (40, 41). Additionally, delivery of heterologous antigens by the type III secretion system (T3SS) in *Y. pseudotuberculosis* and *Salmonella* stimulated antigen-specific cytotoxic T-cell responses, antigen-specific $CD8^+$ memory T cells, and protection against challenge with different pathogens (40, 42, 43). Studies have indicated that both humoral immunity and cellular immunity contribute to vaccine efficacy against plague (21, 24, 44–47). $CD8^+$ T-cell immune responses primed to LcrV appear to confer protection against *Y. pestis* in mice (48, 49). Studies demonstrated that Chinese-origin rhesus macaques immunized with EV76 or mice immunized with the *Y. pestis* Δ*smpB-ssrA* mutant primed a higher anti-F1 IgG titer but an almost undetectable titer with respect to LcrV antigen (38, 50), results that are consistent with other studies of animals immunized with the EV76 or KWC vaccine (51–57). Mice vaccinated with *Y. pestis* KIM5 (lacking *pgm*) generated the CD4 and CD8 T cells that synergistically conferred protection against plague, but T cells from those vaccinated mice could not recognize LcrV (47). Patients who had recovered from plague also barely produced memory T-cell responses to LcrV antigens (58). Pettersson et al. analyzed the localization of LcrV during infection of HeLa cells and were unable to detect any LcrV in the cytosol of the cells (59), but Nilles et al. showed that small amounts of LcrV entered HeLa cells and suggested that this translocation appears to not result from injection via the T3SS (60). So, on the basis of these findings, we hypothesize that a live attenuated *Y. pseudotuberculosis* strain used as a vector to inject the LcrV antigen via the T3SS might prime both antibody responses and specific T-cell responses to LcrV, resulting in enhanced protective immunity to pneumonic plague. In this study, we demonstrated that oral immunization with χ10057(pYA5199) delivering LcrV via the T3SS primed an IgA response to LcrV and specific cellular responses to both LcrV and YpL and provided significant protection against pneumonic plague.

MATERIALS AND METHODS

Media and reagents. Tryptone, yeast extract, tryptose blood agar (TBA), and heart infusion broth (HIB) were from Difco. 2,6-Diaminopimelic acid (DAP) and L-arabinose were from Sigma (St. Louis, MO). Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England BioLabs (Ipswich, MA). *Taq* DNA polymerase (New England BioLabs) was used in all PCR tests. Vent DNA polymerase (New England BioLabs) was used to amplify fragments for cloning. T4 ligase was from Promega (San Luis Obispo, CA). Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, gel-purify fragments, and purify PCR products.

Bacterial strains, plasmids, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. All strains were stored at −70°C in peptone-glycerol. *Escherichia coli* χ6212 was used as an intermediate host for cloning procedures and grown routinely at 37°C in LB broth (67) or on LB solidified with 1.2% Bacto agar (Difco). The *Y. pseudotuberculosis* PB1+ strain provided by Robert Perry (University of Kentucky) and used in this study was grown in LB medium at 27°C. When required, chloramphenicol (Cm; 50 μg/ml), arabinose (0.1%), or 2,6-diaminopimelic acid (DAP; 15 μg/ml) was added. TBA containing 5% sucrose was used for *sacB* gene-based counterselection in allelic-exchange experiments. *Y. pestis* KIM6+(pCD1Ap) was used for challenge studies as previously reported (68). *Y. pestis* cells were grown routinely on Congo red agar from peptone-glycerol stocks and in HIB at 28°C (69). HIB Congo red agar plates were used to confirm the pigmentation (Pgm) phenotype of *Y. pestis* strains (63).

Construction of plasmids. All primers used in this study are listed in Table 2. The *sycE-yopE* (amino acids [aa] 1 to 138) (designated *sycE-yopE*$_{Nt138}$) gene fragment was amplified from *Y. pestis* using primers 1 and 2. The full-length *lcrV* gene was amplified from *Y. pestis* using primers 3 and 4. Then, the *sycE-yopE*$_{Nt138}$ gene fragment was fused with the full-length *lcrV* through overlapping PCR using primers 1 and 4. The fused fragment, *sycE-yopE*$_{Nt138}$*-lcrV*, was cloned into BspEI and HindIII sites of pYA3332 (p15A *ori*) to form plasmid pYA5199 (*yopE*$_{Nt138}$*-lcrV*), which specifies synthesis of $YopE_{Nt138}$-LcrV.

For construction of suicide vectors, primers MsbB1 and MsbB2 and primers MsbB3 and MsbB4 (Table 2) were used for amplifying flanking regions of the *msbB* gene from *Y. pseudotuberculosis* PB1/+, respectively. Then, the DNA fragment containing the Δ*msbB* was amplified using primers MsbB1 and MsbB4 through overlapping PCR. The fused DNA segment (Δ*msbB*) was ligated into the KpnI site of pRE112 to form plasmid pYA5151. In order to replace the *msbB* gene of *Y. pseudotuberculosis* PB1/+ by the *msbB* gene of *E. coli* K-12, the *msbB* gene with its native promoter of *E. coli* K-12 [$P_{msbB\ (EC)}$ *msbB*$_{(EC)}$] was amplified using primers MsbB-K-12-F and MsbB-K-12-R (Table 2). The segment of $P_{msbB\ (EC)}$ *msbB*$_{(EC)}$ was cloned into the PstI and SacI sites between the flanking regions of Δ*msbB* in pYA5151 to form plasmid pYA5152. The segment of ΔP_{crp}::TT *araC* P_{BAD} *crp* was amplified from plasmid pYA4581 (66) using primers Pcrp-F and Pcrp-R (Table 2) and cloned into the KpnI and XmaI sites of pRE112 to form plasmid pYA5153. To delete the *asd* gene (YPTS_3990) from *Y. pseudotuberculosis* PB1/+, primers Asd1 and Asd2 and primers Asd3 and Asd4 (Table 2) were used for amplifying flanking regions of the Δ*asd* gene, respectively. Then, the DNA fragment containing the Δ*asd* was amplified using primers Asd1 and Asd4 through overlapping PCR. The fused flanking region (Δ*asd*) was ligated into the KpnI and XmaI sites of pRE112 to form plasmid pYA5154. All the plasmid constructions were verified through sequencing.

Construction of *Y. pseudotuberculosis* mutants. To replace the *msbB*$_{(Ypt)}$ gene with *msbB*$_{(EC)}$, a *Y. pseudotuberculosis* Δ*msbB868* mutation, which encompassed a 1,295-bp deletion from the 162 bp before the ATG start codon to 170 bp behind the TAG stop codon of *msbB*, was constructed. The suicide plasmid pYA5151 (Δ*msbB*) was conjugationally transferred from *E. coli* χ7213 (62) to the *Y. pseudotuberculosis* PB1/+ wild-type strain. Single-crossover insertion strains were isolated on TBA agar plates containing Cm. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counterselection system (70). The colonies were screened for Cm^s and verified by PCR using

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description or relevant characteristics	Source, derivation, or reference
Strains		
E. coli χ6212	F^- λ^- ϕ80 Δ(*lacZYA-argF*) *endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4*	61
E. coli χ7213	*thi-1 thr-1 leuB6 fhuA21 lacY1 glnV44* Δ*asdA4 recA1* RP4 2-Tc::Mu [λ*pir*]; Km^r	62
Y. pestis KIM6+	Pgm^+, pMT1, pPCP1, pCD1	Robert Perry
Y. pestis KIM6+(pCD1Ap)	Pgm^+, pMT1, pPCP1, pCD1Ap	63
Y. pestis KIM6 (pCD1Ap)	Pgm^-, pMT1, pPCP1, pCD1Ap	Robert Perry
Y. pestis χ10006	Δ*asd Y. pestis* KIM6+	This study
Y. pseudotuberculosis PB1+	Serotype O:1B	Robert Perry
Y. pseudotuberculosis χ10052	Δ*msbB868*	*Y. pseudotuberculosis* PB1+
Y. pseudotuberculosis χ10053	Δ*msbB868*::P_{msbB} *msbB* (EC)	*Y. pseudotuberculosis* χ10052
Y. pseudotuberculosis χ10054	ΔP_{crp21}::TT *araC* P_{BAD} *crp*	*Y. pseudotuberculosis* PB1+
Y. pseudotuberculosis χ10055	Δ*msbB868*::P_{msbB} *msbB* (EC) ΔP_{crp21}::TT *araC* P_{BAD} *crp*	*Y. pseudotuberculosis* χ10053
Y. pseudotuberculosis χ10056	Δ*asd206* ΔP_{crp21}::TT *araC* P_{BAD} *crp*	*Y. pseudotuberculosis* χ10055
Y. pseudotuberculosis χ10057	Δ*asd206* Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*	This study
Plasmids		
pRE112	Suicide vector, Cm^r, mob^- (RP4)R6K *ori*, *sacB*	64
pYA3332	Asd^+; p15A *ori*	65
pYA4581	SD-*crp* and *y3957′* fragments cloned into XhoI/EcoRI sites and PstI/HindIII sites of pYA3700	66
pYA5151	The flanking regions of Δ*msbB* of *Y. pseudotuberculosis* cloned into PstI sites of pRE112	This study
pYA5152	The fragment of Δ*msbB*::$P_{msbB(EC)}$ *msbB*(*EC*) cloned into PstI sites of pRE112	This study
pYA5153	The fragment of ΔP_{crp}::TT *araC* P_{BAD} *crp* from pYA4581 cloned into XmaI and KpnI sites of pRE112	This study
pYA5154	The flanking regions of Δ*asd* of *Y. pseudotuberculosis* cloned into XmaI and KpnI sites of pRE112	This study
pYA5199	The *sycE-yopE′* (aa 1–138)-*lcrV* fragment was cloned into pYA3332	This study
pYA5203	The *sycE-yopE′* (aa 1–138)-*lcrV* fragment and *caf* operon were cloned into pYA3332	This study

[a] Cm, chloramphenicol; Km, kanamycin.

primers MsbB1 and MsbB4. The mutant strain was designated χ10052 (Δ*msbB868*). Then, χ10052 was conjugated with χ7213 harboring the pYA5152 suicide plasmid [Δ*msbB*::$P_{msbB\ (EC)}$ $msbB_{(EC)}$] to transfer Δ*msbB868*::$P_{msbB\ (EC)}$ $msbB_{(EC)}$ into χ10052. Through same screening procedure, mutant strain χ10053 [Δ*msbB868*::$P_{msbB\ (EC)}$ $msbB_{(EC)}$] was confirmed. Then, ΔP_{crp21}::TT *araC* P_{BAD} *crp* was introduced into χ10053 through same procedure to construct mutant strain χ10055 [ΔP_{crp21}::TT *araC* P_{BAD} *crp* Δ*msbB868*::$P_{msbB\ (EC)}$ $msbB_{(EC)}$]. Finally, the Δ*asdA206*-defined deletion was introduced into χ10055 through conjugation of χ7213(pYA5154) (Δ*asdA206*) with χ10055 to form χ10057. The colonies were confirmed for Cm^s and for growth only in the presence of DAP and by PCR using primer set Asd1 and Asd4. All the mutant strains were confirmed by DNA sequence analysis.

Analysis of synthesis and secretion of the recombinant proteins. To analyze the production and secretion of the chimeric proteins through the T3SS, secreted proteins were prepared by using modifications of previously described methods (71). Cultures of *Y. pseudotuberculosis* χ10057 harboring pYA5199 ($yopE_{Nt138}$-*lcrV*) or pYA3332 (empty plasmid) were

TABLE 2 Primers used in this work

Name	Sequence[a]
Primer1	CGGTCCGGAGACATTACTAAGTGAGCGTTGTA (BspEI)
Primer2	GTTTTGTTCGTAGGCTCTAATCATCGTAGCGAACTGATCATGATTTTTCTG
Primer3	GAAAAATCATGATCAGTTCGCTACGATGATTAGAGCCTACGAACAAAACCCA
Primer4	CGGAAGCTTTCATTTACCAGACGTGTCATCTA (HindIII)
MsbB1	CGGGGTACCCGTATTGCGCCGCATAAAGG (KpnI)
MsbB2	CTGAGCTCGGCAGCCTGCAGAGCCATCTACGATGGGCTGACAGACTG
MsbB3	CTCTGCAGGCTGCCGAGCTCAGACGCCGTAAATACATCCATGTAGG
MsbB4	CGGGGTACCTGCGGCAAACCACCTCAAAG (KpnI)
MsbB-K-12-F	CGGGAGCTCTTGAACTTATCATCAGGCGAAGGCCT (SacI)
MsbB-K-12-F	CGGCTGCAGGCTTTCCGGTAATACCGGAC (PstI)
Pcrp-F	CGGCCCGGGCTGATAGATCAACTGCGCGCTCCA (XmaI)
Pcrp-R	CGGGGTACCCTTAACGGGTGCCGTAAACGACGA (KpnI)
Asd-1	CGGGGTACCAGCAACACAGTTGCCGCAATCATCTC (KpnI)
Asd-2	ACGCTATGCGCCGCTAAAAAATAGTGTTTACTGCCCTGCCTTGGAAGG
Asd-3	CAGGGCAGTAAACACTATTTTTTAGCGGCGCATAGCGTGTCATATCGT
Asd-4	CGGCCCGGGCTATAGTATGCCCGTCCGGTTTCATCC (XmaI)

[a] Underlined sequence characters represent the restriction endonuclease sites.

grown overnight in LB broth supplemented with 0.05% arabinose at 26°C and subcultured into calcium-chelated medium (1% tryptone, 0.5% yeast extract, 50 mM MOPS [morpholinepropanesulfonic acid; pH 7.0], 16 mM sodium oxalate, 160 mM magnesium chloride). Cultures were incubated for 6 h at 37°C on a roller drum to provide mild aeration. The optical density at 600 nm (OD_{600}) of cultures was measured, and bacterial cell pellets were collected by centrifugation. The pellets were suspended in SDS loading buffer. The volume of sample buffer was adjusted based on the OD_{600} to normalize the amount loaded on an SDS-PAGE gel. Whole bacterial cells were lysed by heating at 95°C for 5 min. Culture supernatants were concentrated by precipitation with 10% (wt/vol) trichloroacetic acid overnight at 4°C and collected by centrifugation. Pellets were washed with ice-cold acetone and dissolved in 0.05 M Tris-HCl buffer (pH 9.5). Samples were heated at 95°C for 5 min, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. The membranes were probed with rabbit anti-LcrV antibodies as described previously (31).

Translocation of fusion protein $YopE_{Nt138}$-LcrV. The translocation assays were conducted according to procedures described previously (72) with minor modifications. Briefly, HeLa cells (5×10^5) were seeded into 100-mm-diameter tissue culture dishes in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and grown to 80% to 90% confluence. Prior to infection, the cells were washed twice with 5 ml of Dulbecco's phosphate-buffered saline (DPBS). Antibiotic-free medium containing 0.5 μg/ml cytochalasin D was added. Overnight bacterial cultures were diluted 1:20 in HIB and incubated for 30 min at 26°C followed by 1 h at 37°C. HeLa cells were infected with the bacteria at a multiplicity of infection (MOI) of 50 for 4 h at 37°C. Subsequently, the culture medium was removed, and the cells were washed three times with 10 ml DPBS. The HeLa cells were treated with 10 μg/ml proteinase K–D-PBS for 15 min at 37°C to digest secreted but not translocated bacterial protein. Afterward, 3 ml of chilled HBSS containing 2 mM phenylmethylsulfonyl fluoride (Sigma) was added. Cells detached during the proteinase K treatment and were subsequently collected by low-speed centrifugation (600 × *g* for 10 min) and lysed in 1 ml of DPBS containing 0.1% Triton X-100, 10 μg/ml DNase, 10 μg/ml RNase, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% (vol/vol) protease inhibitor (P-8340), and 0.01% (vol/vol) phosphatase inhibitor (P-2850) cocktails (Sigma-Aldrich) and then incubated for 15 min at 4°C. Then, the cell lysates were centrifuged at 12,500 × *g* for 30 min at 4°C, and the pellets (P) obtained, containing the unbroken cells, membranes, and bacteria that had adhered and had been internalized, were resuspended in 200 μl of LDS sample buffer (Pierce ECL, Rockford, IL). The supernatant of the cytoplasmic fraction (C) containing the eukaryotic cytoplasm and the translocated recombinant proteins was filtered through a 0.22-μm-pore-size syringe filter (Millipore), and the proteins were precipitated with 10% trichloroacetic acid and resuspended in 200 μl of LDS sample buffer. Samples were heated at 95°C for 5 min, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. The chimeric proteins were identified using rabbit anti-LcrV antibody followed by peroxidase-conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO). Bound antibodies were detected using an enhanced chemiluminescent detection system (Pierce ECL; Pierce, Rockford, IL).

Animal experiments. Six-week-old female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). All animal procedures were approved by the Arizona State University Animal Care and Use Committee. Mice were acclimated for 1 week after arrival and deprived of food and water for 6 h before starting experiments. The median lethal dose (LD_{50}) of the *Y. pseudotuberculosis* strains in mice was determined according to previous procedures with certain modifications (73–75). Overnight cultures of bacteria were grown at 26°C in LB supplemented with 0.05% arabinose when needed. The next day, 1 ml of this culture was inoculated into 50 ml of the appropriate media and grown with aeration at 26°C to an optical density at 600 nm (OD_{600}) of 1.0. Bacteria were harvested by centrifugation at 24°C and resuspended in 0.5 ml buffered saline solution with gelatin (BSG). Five mice per group were orally inoculated with approximately 10^7, 10^8, or 10^9 CFU of bacteria in 20 μl of BSG by placing a pipette tip behind the incisors of mice. Actual numbers of CFU inoculated were determined by plating serial dilutions onto LB agar. The experiment was repeated one time, and data were combined to calculate the LD_{50}.

To evaluate colonization, 3 mice per group were euthanized on days 3, 6, and 9 after inoculation. Samples of spleen, liver, and Peyer's patches (PPs) were collected and weighed, and BSG buffer (74) was added to reach 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, $\log_{10}$ calculations were applied to the values, and recovery of 0 CFU was reported as 1 CFU/g. The experiment was done twice, and data were combined to calculate colonization titers.

To evaluate the immunogenicity of vaccine strains, strain χ10057 harboring plasmid pYA5199 (*yopE*$_{Nt138}$*-lcrV*) or pYA3332 (empty plasmid) was prepared as described above. Access to food and water was restricted for 6 h, and then 10 mice per group were orally inoculated with 20 μl of BSG containing 1×10^9 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 samples were collected individually after centrifugation. The vaginal tract of each mouse was washed with 100 μl BSG, and the secretory IgA (sIgA) in wash fluids was analyzed individually. The immunogenicity of vaccine strains was evaluated by determining the titers of antibodies in serum or vaginal washes against LcrV (65) and *Y. pestis* whole-cell lysates (YpL) (76) by enzyme-linked immunosorbent assay (ELISA) as described previously (61).

Determination of protective efficacy. A single colony of *Y. pestis* KIM6+(pCD1Ap) was inoculated into HIB medium supplemented with 25 μg/ml ampicillin and grown overnight at 26°C. Bacteria were diluted into 10 ml of fresh HIB enriched with 0.2% xylose and 2.5 mM $CaCl_2$ and supplemented with 25 μg/ml ampicillin to obtain an OD_{620} of 0.1 and incubated at 37°C for intranasal (i.n.) infections (pneumonic plague). Cultures were grown to an OD_{620} of 0.6. The cells were then harvested, and the pellet was resuspended in 1 ml of isotonic PBS. Groups of Swiss Webster mice (10/group) were orally immunized with 10^9 CFU of χ10057 strains containing different plasmids. One group of mice (5/group) was orally vaccinated with BSG as a control. On day 35 after initial immunization, animals lightly anesthetized with a 1:5 xylazine-ketamine mixture were challenged intranasally with 1×10^4 CFU *Y. pestis* KIM6+(pCD1Ap)–20 μl PBS. All infected animals were observed over a 15-day period for the development of signs of plague infection.

Measurement of antibody responses. An enzyme-linked immunosorbent assay (ELISA) was used to assay antibodies to LcrV (77) or YpL (76) in serum. Polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 100 ng/well of purified rLcrV or YpL. Antigens suspended in sodium carbonate bicarbonate coating buffer (pH 9.6) were applied with 100-μl volumes in each well. The coated plates were incubated overnight at 4°C. The procedures for measuring antibody titer were described in our previous report (65). Absorbance readings that were 0.1 higher than PBS control values were considered to represent a positive result.

Analysis of T-cell activation by measuring cytokine production. Spleens taken aseptically from euthanized animals were dissociated using cell strainers (BD Biosciences). The spleen cell suspensions were depleted of red blood cells (RBC) using RBC lysis buffer (Sigma), and splenocytes were extensively washed with cold PBS. Cells resuspended in RPMI 1640 plus GlutaMAX (Gibco) supplemented with 5% fetal bovine serum and 100 μg/ml penicillin-streptomycin were seeded in 96-well plates (1×10^6/well) and stimulated with YpL antigen (4 μg/ml), the LcrV antigen (4 μg/ml), or concanavalin A (ConA) (Sigma) (1 μg/ml) as a positive control. After 3 days, the supernatant was collected and measured for cytokine content using a multiplex assay with BioPlex (Bio-Rad).

Statistical analysis. The log-rank test was used for analysis of the survival curves. Data are expressed as means ± standard deviations (SD). Two-way analysis of variance (ANOVA) was used for antibody titer and cytokine analysis. The Student *t* test was used to compare bacterial loads in challenged mouse groups. A P value of <0.05 was considered significant.

RESULTS

Construction of a live attenuated *Y. pseudotuberculosis* strain as a carrier for delivering antigen. Rebeil at al. demonstrated that *Y. pseudotuberculosis* PB1 produces penta-acylated and C16:1-containing hexa-acylated lipid A species when grown at 21°C, while tetra-acylated lipid A and C16:0-containing penta-acylated lipid A are observed when the same strain is grown at 37°C (78). The temperature-sensitive difference in lipid A acylation results in *Y. pseudotuberculosis* has not yet been elucidated. *Y. pseudotuberculosis* contains a biochemically uncharacterized *lpxL* homolog that may be responsible for formation of penta-acylated lipid A at 21°C and/or 37°C. Additionally, Pérez-Gutiérrez et al. (79) have shown that the *Y. enterocolitica* MsbB acyltransferase is temperature sensitive and has a high (94%) degree of identity with MsbB (YPTS_2105) of *Y. pseudotuberculosis*. Therefore, we infer that MsbB in *Y. pseudotuberculosis* is temperature sensitive.

The major tetra-acylated lipid A at 37°C (mammalian host temperature) formed in *Y. pestis* can suppress early immune responses (80, 81). In order to overcome the immunosuppression, *Y. pestis* was engineered to produce hexa-acylated lipid A by expressing *E. coli* LpxL (68, 80). This strain was attenuated and could induce potent protective immunity to plague (68, 80). Our studies showed that combining production of hexa-acylated lipid A and regulation of Crp synthesis under the control of a *araC* P_{BAD} regulon made the *Y. pestis* mutant more attenuated than *Y. pestis* with hexa-acylated lipid A alone while retaining great immunogenicity (68). Here, we used an approach to achieve *Y. pseudotuberculosis* attenuation similar to that described in our previous paper (68). Unlike *Y. pestis*, *Y. pseudotuberculosis* encodes a functional *lpxL* homolog (82). In addition, MsbB in *Y. pseudotuberculosis* may be temperature sensitive, resulting in major production of tetra-acylated lipid A observed in *Y. pseudotuberculosis* grown at 37°C and associated with immunosuppressive properties (78). Therefore, we replaced only the *msbB* gene and its promoter in *Y. pseudotuberculosis* with the *E. coli msbB* gene and its native promoter [Δ*msbB*:: P_{msbB}::$msbB_{(EC)}$] to drive the mutant strain to synthesize hexa-acylated lipid A at 37°C, which can be recognized by Toll-like receptor 4 (TLR4) to enhance immunostimulatory properties. We thus constructed χ10053 [Δ*msbB*::P_{msbB} $msbB_{(EC)}$]. Then, the ΔP_{crp21}::TT *araC* P_{BAD} *crp* mutation was introduced into strain χ10053 to construct χ10055 [Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*] to obtain much attenuation. Measurement of bacterial growth indicated that the growth curve of the mutant strain with the Δ*msbB868* or Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ mutation was the same as that of the wild-type strain, while mutant strain χ10055 also containing the ΔP_{crp21}::TT *araC* P_{BAD} *crp* mutation required arabinose for optimal growth (Fig. 1A).

Lipopolysaccharide (LPS) synthesis of each mutant was analyzed by silver staining (Fig. 1B). The result demonstrated that there was no detectable alteration in the lipid A acylation pattern of wild-type *Y. pseudotuberculosis* PB1+ that was dependent on growth temperature. Surprisingly, the lipid A portion was not altered even in the *msbB* (*YPTS_2105*) mutant strain. In addition, replacing the *Y. pseudotuberculosis msbB* gene and its promoter with the *E. coli msbB* gene and its native promoter [Δ*msbB*:: P_{msbB}::$msbB_{(EC)}$] also did not cause any shifts in the migration of LPS as analyzed by silver staining (Fig. 1B). The lipid A structures of wild-type *Y. pseudotuberculosis* PB1+, χ10052 (Δ*msbB*), χ10053 [Δ*msbB*:: P_{msbB}::$msbB_{(EC)}$], and χ10055 [Δ*msbB*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*] were further analyzed by matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS). However, as has been previously reported (78, 83), the data were extremely complex (see Fig. S1 in the supplemental material). Although MALDI-MS indicated the presence of a complex mixture of lipid A structures, we observed that all strains synthesized penta-, hexa-, and hepta-acylated lipid A species at 37°C with minor temperature-dependent variations in the proportions of the major lipid A species. Consistent with the activity of MsbB in *Y. pestis*, minor peaks corresponding to the proposed lauroyl (C12)-containing lipid A molecules were absent in the Δ*msbB* mutant relative to the wild type. No significant changes in the acylation state were observed in the lipid A population obtained from Δ*msbB* compared to Δ*msbB*::P_{msbB}::$msbB_{(EC)}$ mutants (see Fig. S1).

Virulence of χ10055 determined by oral administration in Swiss Webster mice demonstrated that the LD_{50} of χ10055 increased at least by 10^2-fold ($>4 \times 10^9$ CFU) compared to that of the wild-type strain ($LD_{50} = 5 \times 10^7$ CFU). But we did not observe any changes in the virulence of χ10052 (Δ*msbB868*) and χ10053 [Δ*msbB868*::P_{msbB}::$msbB_{(EC)}$] (data not shown). To facilitate antigen synthesis specified by plasmids in recombinant *Y. pseudotuberculosis* strains, we adapted the balanced-lethal Asd^+ plasmid maintenance system developed for *Salmonella* (84) in *Y. pseudotuberculosis*. Based on the attenuated χ10055 mutant, the Δ*asd* mutation was introduced into χ10055 to construct χ10057 [Δ*asd-206* Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*], which was used as a carrier for delivering antigen (Fig. 1C).

Synthesis, secretion, and translocation of recombinant chimeric $YopE_{Nt138}$-LcrV proteins by strain χ10057. Cellular immunity plays an important role in protection against pneumonic plague (45, 46, 85–88). To stimulate cellular immunity, heterologous antigens fused with YopE (aa 1 to 138) allow the chimeric protein to be specifically transported via the T3SS of live attenuated *Y. pseudotuberculosis* strains to become accessible to the major histocompatibility complex (MHC) class I-restricted antigen-processing pathways and stimulate an antigen-specific cellular immune response (40, 41). Thus, we constructed plasmid vectors containing the secretion and translocation signals of the *Yersinia* T3SS YopE effector protein, which are specified by the first 138 amino acids in the amino-terminal region of YopE (designated $YopE_{Nt138}$). SycE encoded by *sycE* is the chaperon protein for YopE and increases the translocation of YopE fusion protein (89) or $YopE_{Nt138}$ fusion protein (43, 90). The *lcrV* of *Y. pestis* generated by in-frame fusion with *sycE-yopE*$_{Nt138}$ was cloned into pYA3332 to generate pYA5199 (Fig. 2A).

To test secretion of the chimeric $YopE_{Nt138}$-LcrV protein, cultures of χ10057 harboring either pYA3332 (plasmid control) or pYA5199 (*yopE*$_{Nt138}$-*lcrV*) were grown at 26°C to an OD_{600} of 0.8 in LB medium with 0.05% arabinose and then subcultured into calcium-chelated medium supplemented with 0.05% arabinose for 6 h at 37°C as described in Materials and Methods. Immunoblotting was used to detect synthesis of $YopE_{Nt138}$-LcrV fusion protein. The molecular mass of $YopE_{Nt138}$-LcrV was 52 kDa as expected

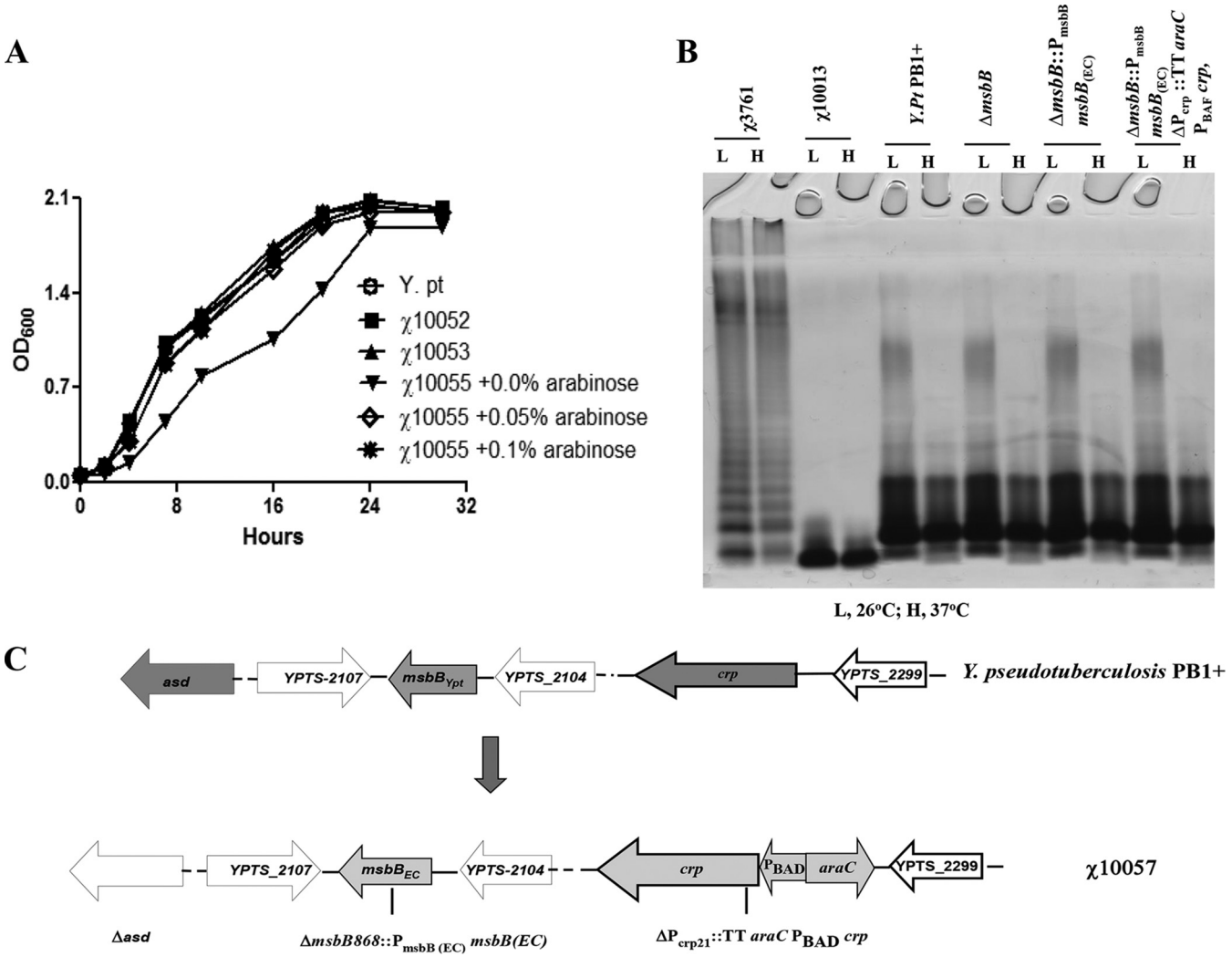

FIG 1 LPS phenotypes, growth curves of mutant and parent strains, and schematic diagram of strain constructions. (A) The growth curves of *Y. pseudotuberculosis* PB1+ (Y. pt), χ10052, χ10053, and χ10055 in LB medium supplemented with different amounts (0%, 0.05%, and 0.1%) of arabinose at 26°C. (B) LPS was visualized by the use of silver-staining polyacrylamide gel electrophoresis (PAGE) gels. The strains (from left to right) are as follows: χ3761 (*Salmonella enterica* serovar Typhimurium UK-1), χ10013 (Δ*lpxP32 Y. pestis* KIM6+), wild-type *Y. pseudotuberculosis* PB1+ (*Y. pt* PB1+), χ10052 (Δ*msbB868*), χ10053 [Δ*msbB868*::P_{msbB} $msbB_{(EC)}$], and χ10055 [Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*]. All strains were cultured at 26°C and 37°C. (C) Schematic diagram depicting the chromosomal structure of χ10057 [Δ*asd206* Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*].

(Fig. 2B). These results demonstrated that the $YopE_{Nt138}$-LcrV hybrid proteins were synthesized and secreted *in vitro*.

To analyze translocation of the hybrid proteins, HeLa cells were infected with χ10057 harboring either pYA3332 as a negative control or pYA5199 (*yopE*$_{Nt138}$-*lcrV*). Our results demonstrated that $YopE_{Nt138}$-LcrV was translocated into the cytosol of host cells by the *Y. pseudotuberculosis* mutant strain (Fig. 2C).

Virulence and persistence of recombinant strains harboring different plasmids in mice. To investigate whether plasmids introduced into the χ10057 strain [Δ*asd-206* Δ*msbB868*::P_{msbB} $msbB_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp*] affect its virulence, we infected Swiss Webster mice orally with 4.5×10^9 CFU of χ10057(pYA3332) (vector control) or 7.0×10^9 CFU of χ10057(pYA5199) (*yopE*$_{Nt138}$-*lcrV*). All the mice infected with χ10057 harboring different plasmids did not show any sign of weight loss or any symptoms of disease. Thus, the LD_{50}s of χ10057(pYA3332) and χ10057(pYA5199) were more than 4.5×10^9 CFU. In addition, our results demonstrated survival of 6 of 10 mice infected with 1.5×10^7 CFU of wild-type *Y. pseudotuberculosis* PB1+, 4 of 10 mice infected with 1.5×10^8 CFU of *Y. pseudotuberculosis* PB1+, and 1 of 10 mice infected with 1.5×10^9 CFU of *Y. pseudotuberculosis* PB1+. Therefore, the LD_{50} of wild-type *Y. pseudotuberculosis* PB1+ was around 1.5×10^8 CFU. On the basis of these data, we evaluated the ability of the χ10057 strain harboring different plasmids to disseminate into Peyer's patches, spleens, and livers of mice. We orally infected groups of mice with 0.7×10^9 CFU of wild-type *Y. pseudotuberculosis* PB1+, 1.5×10^9 CFU of χ10057(pYA3332), or 1.3×10^9 CFU of χ10057(pYA5199).

In the Peyer's patches, the titers of wild-type *Y. pseudotuberculosis* PB1+ strain steadily increased at 3 and 6 days postinfection and slightly declined at 9 days postinfection. The titers of χ10057(pYA3332) and χ10057(pYA5199) strains were significantly lower than that of the wild-type PB1+ strain at different

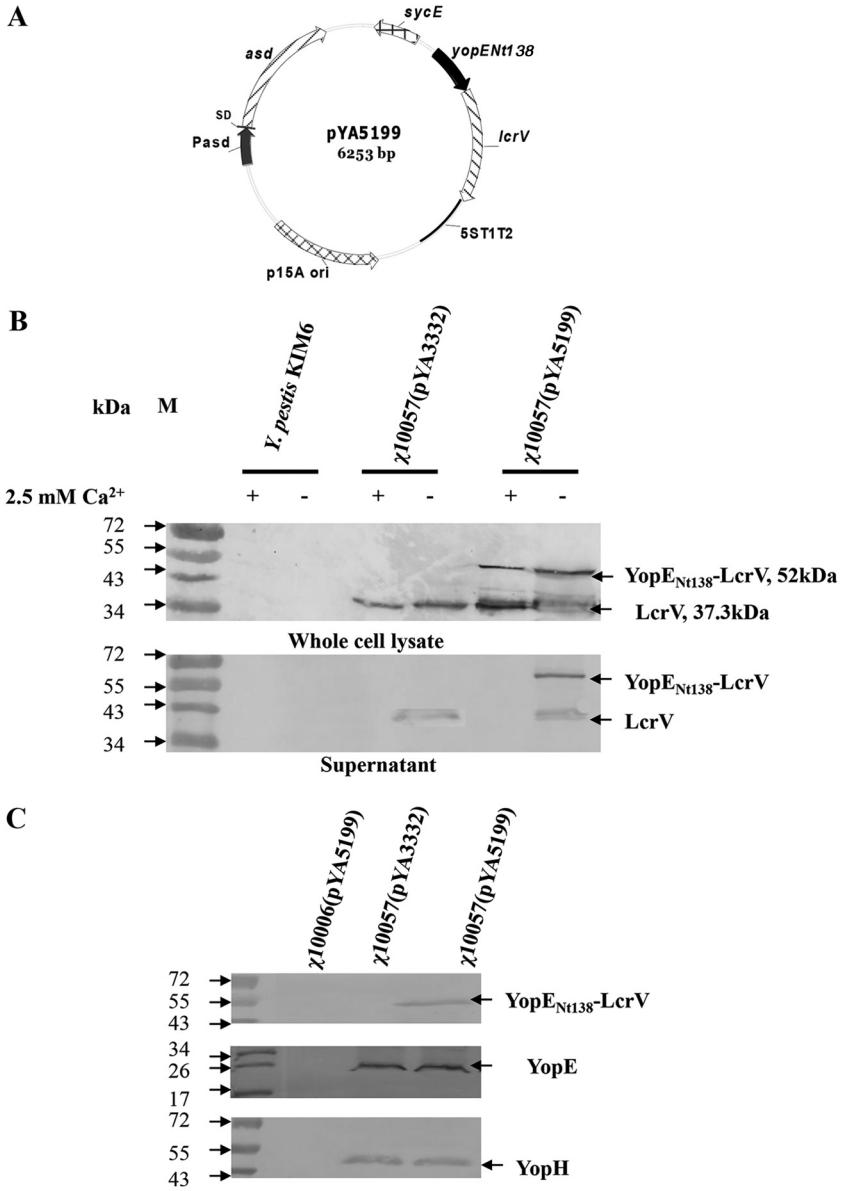

FIG 2 Synthesis, secretion, and translocation of Yop_{ENt138}-LcrV in χ10057(pYA5199). (A) Physical map of pYA5199. (B) The synthesis and secretion of $YopE_{Nt138}$-LcrV were determined in χ10057(pYA5199) by Western blotting. *Y. pestis* KIM6+ (no-pCD1 plasmid) was used as the negative control and χ10057(pYA3332) as the vector control. (C) Translocation of $YopE_{Nt138}$-LcrV into the cytosol of HeLa cells infected by χ10057(pYA5199) was detected by the use of an enhanced chemiluminescent detection system. χ10006(pYA5199) (Δ*asd Y. pestis* KIM6+) was used as the negative control and χ10057(pYA3332) as the vector control; translocation of YopE and YopH was used as the interior control.

time points postinfection (Fig. 3A). The bacterial titers of χ10057(pYA5199) significantly increased, while the titers of χ10057(pYA3332) decreased around 1 log at 6 days postinfection. At 9 days postinfection, the bacterial titers of the two mutant strains reached similar levels (Fig. 3A). The wild-type strain colonized effectively in the spleen and liver and reached higher titers at 3, 6, and 9 days postinfection, but the mutant strain containing different plasmids was unable to effectively disseminate into spleen and liver at 3 days postinfection (Fig. 3B and C). Although the titers of χ10057(pYA5199) in spleens and livers were significantly lower than those of the wild-type strain, strain χ10057(pYA5199) synthesizing $YopE_{Nt138}$-LcrV seemed to be more effective in colonizing the spleen and liver than χ10057(pYA3332) at 6 days postinfection (Fig. 3B and C). At 9 days postinfection, the titers of χ10057(pYA3332) and χ10057(pYA5199) in the spleen and liver were very low (Fig. 3B and C). At 15 days postinfection, no bacteria are detected in the spleens and livers of mice infected with χ10057(pYA3332) and χ10057(pYA5199) (data not shown). The results suggested that χ10057(pYA5199) synthesizing $YopE_{Nt138}$-LcrV might increase its ability to invade the spleen and liver at 6 days postinfection.

Efficacy of protection against pneumonic plague challenge. The LD_{50} of *Y. pestis* KIM6+(pCD1Ap) used as a challenge strain was 100 CFU for i.n. challenge (68). Groups of mice were orally immunized with a single dose (10^9 CFU) of χ10056(pYA3332), χ10057(pYA3332) (plasmid control), χ10056(pYA5199), or

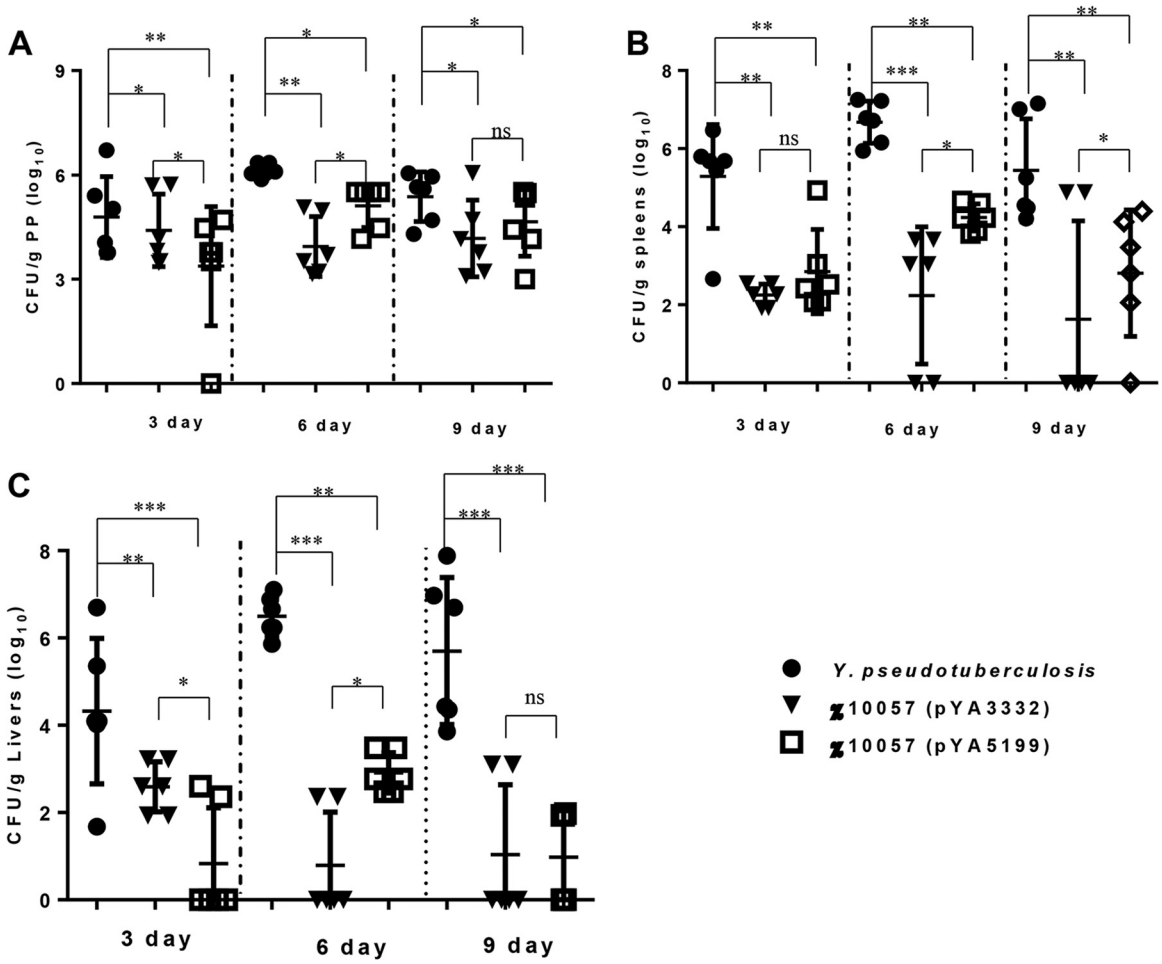

FIG 3 Kinetics of bacterial burden in mice infected orally with *Y. pseudotuberculosis* PB1+, χ10057(pYA3332), or χ10057(pYA5199). Groups of mice were inoculated orally with 0.7×10^9 CFU of wild-type *Y. pseudotuberculosis* PB1+, 1.5×10^9 CFU of χ10057(pYA3332), or 1.3×10^9 CFU of χ10057(pYA5199). CFU counts 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. The experiment was performed twice with similar results, and the results were pooled for presentation.

χ10057(pYA5199) (*yopE*$_{Nt138}$-*lcrV*) or with BSG as a negative control or were subcutaneously immunized with 2.5×10^7 CFU of *Y. pestis* KIM5 (Pgm$^-$) as a standard attenuated *Y. pestis* vaccine and were challenged intranasally with ~240 LD_{50} (2.4×10^4 CFU) of *Y. pestis* KIM6+ (pCD1Ap) at 35 days after the initial immunization. Results showed that a single oral dose of χ10057(pYA5199) provided 80% protection against an i.n. KIM6+(pCD1Ap) challenge (Fig. 4). Partial protection was provided by immunization with *Y. pestis* KIM5 (Pgm$^-$) (60%), χ10056(pYA3332) (42%), χ10056(pYA5199) (40%), or χ10057(pYA3332) (40%). Their protective efficacy was significantly lower than that of χ10057(pYA5199). None of the mice immunized with BSG were protected (Fig. 4).

The mice surviving wild-type *Y. pseudotuberculosis* PB1+ infection were also pooled (10 mice) for intranasal challenge with ~130 LD_{50} (1.3×10^4 CFU) of *Y. pestis* KIM6+ (pCD1Ap). Only 3 mice survived pneumonic challenge during 15 days of observation.

Antibody responses in mice orally immunized with the recombinant *Y. pseudotuberculosis* strains. Measurement of total IgG responses to YpL indicated that the levels of anti-YpL titers of χ10057(pYA3332) and χ10057(pYA5199) were similar by week 2 and were boosted by week 4 to levels similar to those of the anti-YpL antibodies (Fig. 5A). The anti-YpL titers from both strains were significantly higher than those from the BSG-immunized group ($P < 0.001$) (Fig. 5A). The IgG titers of anti-LcrV were slightly higher in mice immunized with χ10057(pYA5199) than in those immunized with χ10057(pYA3332) by week 2 (Fig. 5B). The anti-LcrV titers induced by χ10057(pYA3332) and χ10057(pYA5199) were boosted to similar levels in mice immunized with either strain by week 4.

Live attenuated vaccines administered by the oral route can generate mucosal immune responses, in addition to effective development of humoral immune responses (91). Here, we looked at secretory IgA (sIgA) to LcrV in vaginal washes of mice orally immunized with χ10057(pYA5199) and χ10057(pYA3332). The measurement of sIgA indicated that only χ10057(pYA5199)-immunized mice produced significant levels of anti-LcrV IgA at week 2 ($P < 0.01$) and week 4 ($P < 0.001$), while the χ10057(pYA3332)- or BSG-immunized mice did not produce any levels of anti-LcrV IgA (Fig. 5C).

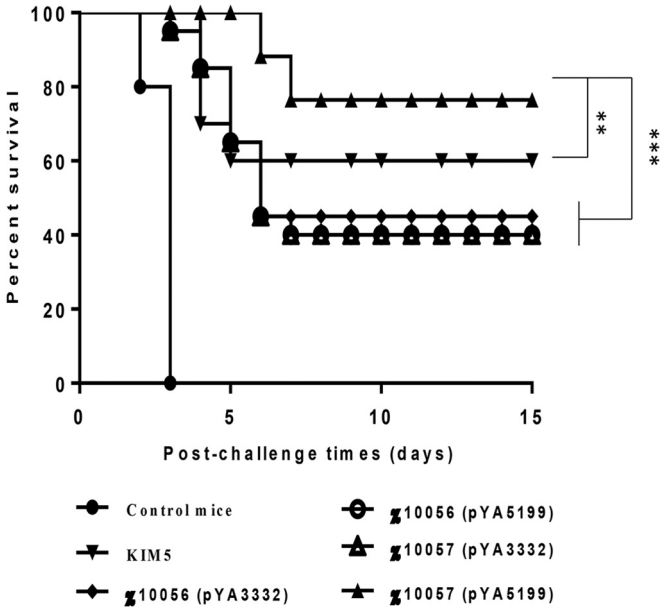

FIG 4 Mouse survival after *Y. pestis* KIM6+(pCD1Ap) challenge. Swiss Webster mice were orally vaccinated with a dose of 2.0×10^9 CFU of χ10056(pYA3332), 2.5×10^9 CFU of χ10056(pYA5199) as the plasmid control, 2.6×10^9 CFU of χ10057(pYA3332), or 2.2×10^9 CFU of χ10057(pYA5199) or with BSG as the negative control or were subcutaneously immunized with 2.5×10^7 CFU of *Y. pestis* KIM5 (Pgm^-) as a standard attenuated *Y. pestis* vaccine. Mice were challenged at 35 days after initial vaccination with 2.4×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap) via the i.n. route. Survival of immunized mice was significantly greater than that of the PBS controls in all experiments (****, $P < 0.0001$). Survival of mice immunized with χ10057(pYA5199) was significantly greater than that of mice immunized with strains χ10056(pYA3332), χ10056(pYA5199), and χ10057(pYA3332) (***, $P < 0.001$) and with *Y. pestis* KIM5 (**, $P < 0.01$). In each experiment, there were 10 mice in the vaccinated group and 5 mice in the control group. All experiments were performed twice with similar outcomes, and the results were pooled for presentation.

We also measured titers of IgG subtypes (IgG1 and IgG2a) in sera from mice immunized with χ10057(pYA3332) and χ10057(pYA5199). The levels of anti-YpL IgG1 and IgG2a isotype antibodies rapidly increased after vaccination at 2 weeks and gradually increased at 4 weeks. At 2 and 4 weeks postimmunization with χ10057(pYA3332), the ratios of the level of IgG1 to YpL to that of IgG2a to YpL were 0.65:1 and 0.7:1, respectively. Similarly, the ratios of the level of IgG1 to YpL to that of IgG2a to YpL were 0.6:1 and 0.65:1 in mice at 2 and 4 weeks postimmunization with χ10057(pYA5199), respectively (Fig. 6A and B). The results indicated that the Th1-biased response to YpL was primed by both χ10057(pYA3332) and χ10057(pYA5199). The levels of anti-LcrV IgG1 and IgG2a antibodies also rapidly increased after vaccination at 2 weeks and were boosted slightly at 4 weeks postimmunization. But the ratio of IgG1 to IgG2a was very close to 1 in mice immunized with χ10057(pYA3332) or χ10057(pYA5199) (Fig. 6C and D).

Cellular immune response in mice orally immunized with χ10057(pYA5199). Several reports have demonstrated that protection against pneumonic plague requires cell-mediated immunity induced by IFN-γ and TNF-α (45, 46). IL-17 also contributes to cell-mediated defense against pulmonary *Y. pestis* infection (92). So, we looked at these three important cytokines. To evaluate cellular immune responses by examining production of IFN-γ, TNF-α, and IL-17 after immunization, we orally vaccinated Swiss Webster mice (4/group) with 10^9 CFU of χ10057(pYA3332) or χ10057(pYA5199) and with BSG as a negative control. At 21 days after the initial vaccination, splenocytes isolated from mice immunized with BSG, χ10057(pYA3332), or χ10057(pYA5199) were stimulated for 72 h with 4 μg/ml of LcrV, 4 μg/ml of YpL, 1 μg/ml of concanavalin A (ConA) as a positive control, or media (RPMI 1640) as a negative control. The supernatants of the cultures were collected and analyzed using a mouse multiplex assay with BioPlex (Bio-Rad). Results showed that splenic cells from mice vaccinated with χ10057(pYA5199) produced levels of IFN-γ, TNF-α, and IL-17 in response to restimulation with the LcrV antigen that were significantly higher than those observed for χ10057(pYA3332), while cells from the BSG-immunized mice did not produce these cytokines (Fig. 7). These results suggested that immunization with a *Y. pseudotuberculosis* strain delivering LcrV via the T3SS could elicit an LcrV-specific cellular immune response. Splenic cells from mice vaccinated with χ10057(pYA5199), χ10057(pYA3332), or BSG also produced cytokine profiles similar to those seen with YpL restimulation, but the levels of IFN-γ, TNF-α, and IL-17 produced by YpL stimulation were significantly lower than those produced by LcrV stimulation (Fig. 7). In addition, production of IL-4 in supernatants was measured, but the levels of IL-4 were very low (see Fig. S4A in the supplemental material). We compared the levels of IFN-γ with those of IL-4 in the supernatant of splenic cells stimulated with LcrV *in vitro*. The ratio of IFN-γ to IL-4 determined for the mouse group subjected to χ10057(pYA5199) immunization was significantly higher than the ratio seen with mice subjected to immunization with BSG and χ10057(pYA3332) (see Fig. S4B). The results suggested that the immune response elicited by immunization of χ10057(pYA5199) had a Th1 bias.

DISCUSSION

Our MALDI-MS analysis suggested that the *msbB* (*YPTS_2105*) deletion did not significantly alter the major lipid A structures observed in *Y. pseudotuberculosis*. Protein alignment indicated that YPTS_2105 and MsbB of *E. coli* K-12 are 65% identical and that YPTS_2105 and MsbB of *Y. pestis* are 99% identical. D'Hauteville et al. showed that *Shigella flexneri* had two functional *msbB* genes, one carried by the chromosome (*msbB1*) and the other by the virulence plasmid (*msbB2*), the products of which act in complement to produce full acyl-oxy-acylation of the myristate at the 3′ position of the lipid A glucosamine disaccharide (93). We assumed that *Y. pseudotuberculosis* PB1+ might have another *msbB* gene elsewhere, but no extracted gene with high identity to the *msbB* of *E. coli* or *Y. pestis* was identified as a result performing a deep BLAST search among the genomes of *Y. pseudotuberculosis* PB1+, *Y. pestis* KIM, and *E. coli* K-12. Thus, it is possible that *lpxP* might be responsible for providing hexa-acylation of lipid A in *Y. pseudotuberculosis* PB1+. Currently, the MS data do not convincingly support the hypothesis that *YPTS_2105* encodes a functioning MsbB; however, at least three minor peaks corresponding to lauroyl (C12)-modified lipid A were absent in Δ*msbB* lipid A mixtures. Our results showing that hexa-acylated lipid A was predominant in *Y. pseudotuberculosis* PB1+ at 37°C are inconsistent with previous reports (78). In addition, using a different strain and different growth conditions, Montminy also showed that *Y. pseudotuberculosis* IP266 contained predominately tetra-, penta-, and

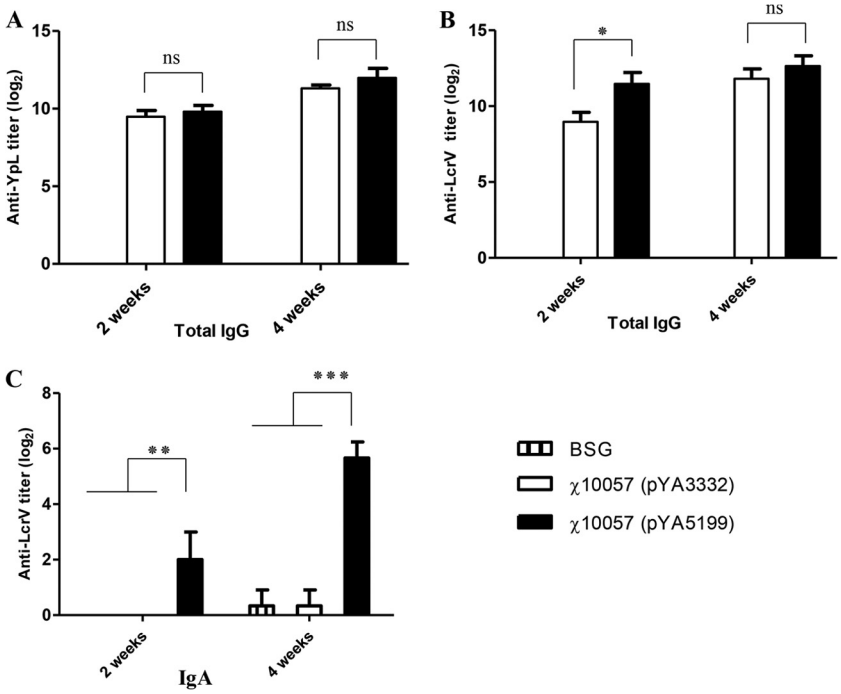

FIG 5 Antibody responses in sera and vaginal washes of mice immunized with BSG, χ10057(pYA3332), or χ10057(pYA5199). (A) Total IgG response to *Y. pestis* KIM5+ whole-cell lysate (YpL). (B) Total IgG response to recombinant LcrV. (C) The secretory IgA response to LcrV. The sera from 12 mice were individually analyzed, and the experiments were performed twice with consistent results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

hexa-acylated lipid A species when grown at 37°C (94). These contradictions may have been caused by differences in the bacterial strains and/or growth conditions used. A complete examination of the genetic basis for the complex multitude of modified lipid A molecules observed in *Y. pseudotuberculosis* is beyond the scope of this report and will be performed in a subsequent study.

Mucosal exposure to infectious agents and other foreign antigens often results in the development of mucosal and serum antibodies and cell-mediated immune responses. Delivering specific vaccine antigens through mucosal immunization remains an attractive approach for immunization against infections, especially those acquired at mucosal surfaces. Induction of IgA responses provides specific protection against many respiratory, enteric, and genital infections (91). As we know, recombinant attenuated *Salmonella* vaccines (RASVs) have been used mostly to induce mucosal and systemic immunity either to *Salmonella* itself or to heterologous antigens delivered by RASVs (95–97). Live attenuated *Y. pseudotuberculosis* strains as oral vaccines against different pathogens, including those causing plague, have been explored (36–40).

The stimulation of mucosal immunity and adaptive immunity was expected to result in protection against infection starting at other mucosal surfaces (98) and thus to potentially protect against pneumonic plague. Our results indicated that χ10057 harboring different plasmids was highly attenuated but still could temporarily colonize in the gut and internal organs of mice for more than a week. However, the protective efficacy of χ10057(pYA5199) was significantly better than that of χ10057(pYA3332). Antibody responses indicated that the titers of total IgG to YpL and LcrV primed in mice immunized with χ10057(pYA3332) and χ10057(pYA5199) did not show any significant difference (Fig. 5A and B) but that the titers of IgA to LcrV primed from χ10057(pYA5199) immunization were significantly higher than that induced by χ10057(pYA3332) (Fig. 5C). Thus, the anti-LcrV IgA production observed in mice vaccinated with χ10057(pYA5199) ($yopE_{Nt138}$-*lcrV*) may contribute higher protection against pneumonic plague and can be associated with $yopE_{Nt138}$-*lcrV* expression in χ10057(pYA5199). Tjärnlund et al. have indicated that actively secreted IgA plays a role in protection against mycobacterial infections in the respiratory tract and modulates the proinflammatory response induced by mycobacteria (99). Furuya et al. also suggested that IgA indirectly affects adaptive T-cell immunity through modulating the homeostasis of the mucosal surface (100). Currently, it is unclear whether the IgA produced by immunization with χ10057(pYA5199) might be associated with elicitation of a proinflammatory response and might influence T-cell immunity. We are planning to conduct investigations to test this hypothesis.

Additionally, compared to those from mice orally vaccinated with χ10057(pYA3332), splenocytes from mice orally vaccinated with χ10057(pYA5199) produced high levels of cytokines such as IFN-γ, TNF-α, and IL-17 upon restimulation with both LcrV antigen and YpL (*Y. pestis* whole-cell lysate) *in vitro* (Fig. 7), which indicated the development of LcrV-specific and *Y. pestis*-specific cellular immune responses in mice vaccinated with χ10057(pYA5199) ($yopE_{Nt138}$-*lcrV*). As indicated in Fig. 7, we also observed that splenic cells from vaccinated mice produced significantly lower levels of IFN-γ, TNF-α, and IL-17 upon YpL restimulation than were seen upon LcrV stimulation (Fig. 7). This could have been due to the method of preparation of YpL, since YpL was made from a lysate of *Y. pestis* KIM+(pCD1Ap) cultured overnight in the calcium-chelated HIB medium at 37°C (76). As

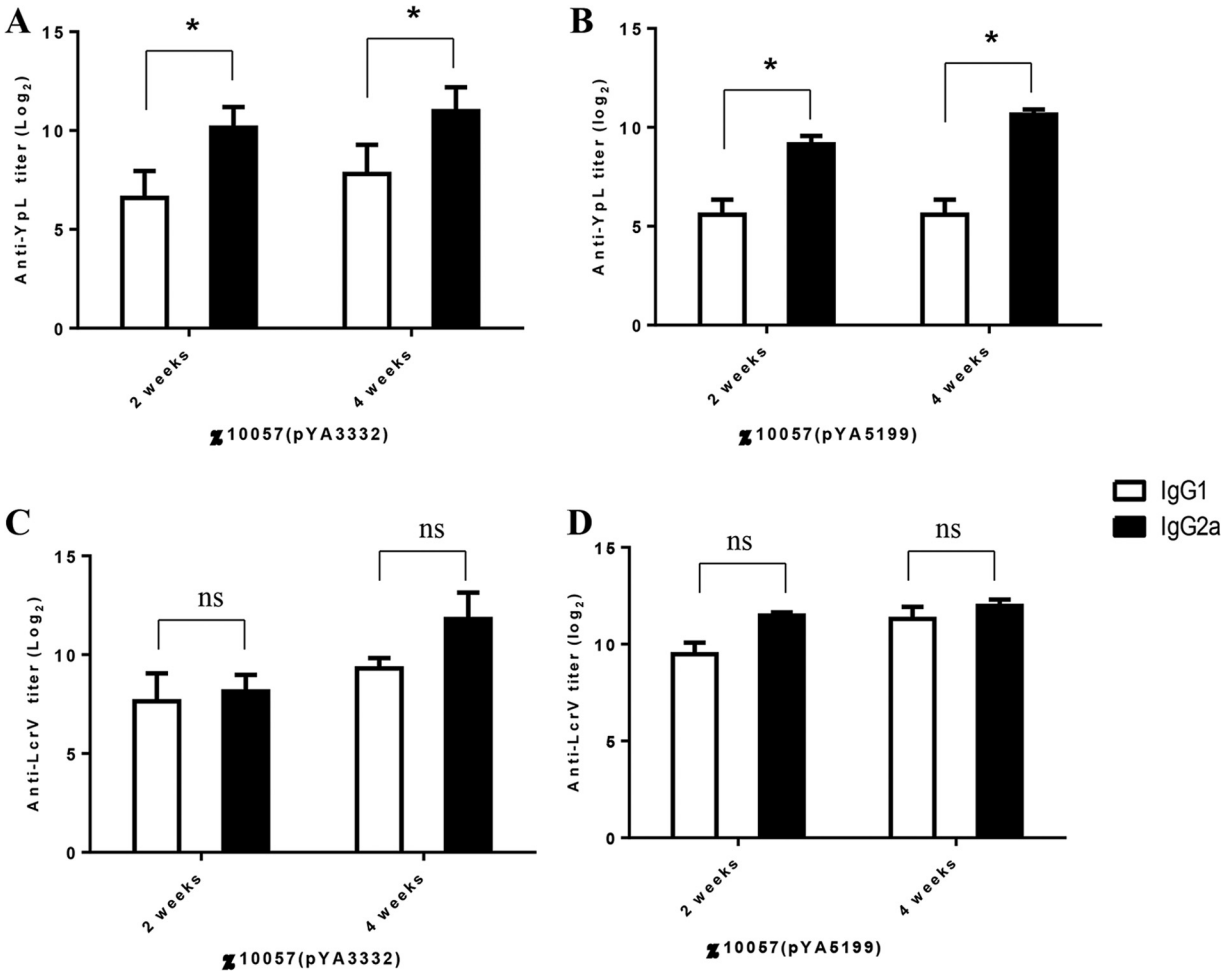

FIG 6 Serum IgG1 and IgG2a responses to YpL and recombinant LcrV. (A) Levels of IgG1 and IgG2a antibody to YpL in sera of mice orally immunized with χ10057(pYA3332). (B) Levels of IgG1 and IgG2a antibody to YpL in sera of mice orally immunized with χ10057(pYA5199). (C) Levels of IgG1 and IgG2a antibody to recombinant LcrV in sera of mice orally immunized with χ10057(pYA3332). (D) Levels of IgG1 and IgG2a antibody to recombinant LcrV in sera of mice orally immunized with χ10057(pYA5199). The sera from 12 mice were individually analyzed, and the experiments were performed twice with consistent results. *, $P < 0.05$.

we know, tetra-acylated lipid A of *Y. pestis* KIM6+(pCD1Ap) predominated at 37°C (68, 78, 101–103). Thus, predominant tetra-acylated lipid A in *Y. pestis* whole-cell lysate prepared under this condition inhibited the induction of proinflammatory cytokines and type I interferons (80), which might result in lower levels of IFN-γ, TNF-α, and IL-17 production.

Derbise et al. indicated that introduction of the pGEN-*caf* plasmid (low-copy-number plasmid) expressing F1 antigen into an attenuated *Y. pseudotuberculosis* strain V674 to form V674 pF1 greatly enhanced protective efficacy (39). There are significant differences between our study and that by Derbise et al., such as the use of different *Y. pseudotuberculosis* strains (PB1+ versus IP32953), different mouse species (Swiss Webster versus OF1), different challenge strains [KIM6+(pCD1Ap) versus CO92], and different mutations [Δ*msbB868*::P_{msbB} *msbB*$_{(EC)}$ ΔP_{crp21}::TT *araC* P_{BAD} *crp* versus ΔHPI Δ*yopK* Δ*psaA*] and the harboring of different recombinant plasmids (pYA5199 versus pGEN-*caf*). However, the encapsulated *Y. pseudotuberculosis* V674 pF1 strain, which provided complete protection against intranasal challenge with 10^5 CFU of *Y. pestis* CO92 (39), was more efficacious than χ10057(pYA5199) (*yopE*$_{Nt138}$-*lcrV*), which provided 80% protection against intranasal challenge with 2.4×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap). In our study, we also cloned the *caf* operon into pYA5199 to form pYA5203. χ10057 harboring pYA5203 produced and secreted levels of YopE$_{Nt138}$-LcrV similar to those seen with the strain containing pYA5199 and also synthesized high levels of F1 antigen under conditions of culture temperatures of 37°C and 26°C, which affected the bacterial growth rate (data not shown). Animal experiments demonstrated that no colonization and no protection were observed in the spleens and livers of mice immunized with χ10057(pYA5203) delivering YopE$_{Nt138}$-LcrV/F1 (data not shown). Our observations indicated that overexpressing *caf1* in live attenuated *Y. pseudotuberculosis* strains affected the growth rate of bacteria, weakened the bacterial resistance in mice, and decreased immunogenicity. Cao et al. also demonstrated that overexpressing *caf1* in wild-type *Salmonella enterica* serovar Typhimurium greatly attenuated the virulence of the *Salmonella in vivo* (104). Thus, we are trying to reduce *caf1* expression to optimal levels in the same host strain, χ10057, in which synthesizing optimal levels of F1 antigen and LcrV simul-

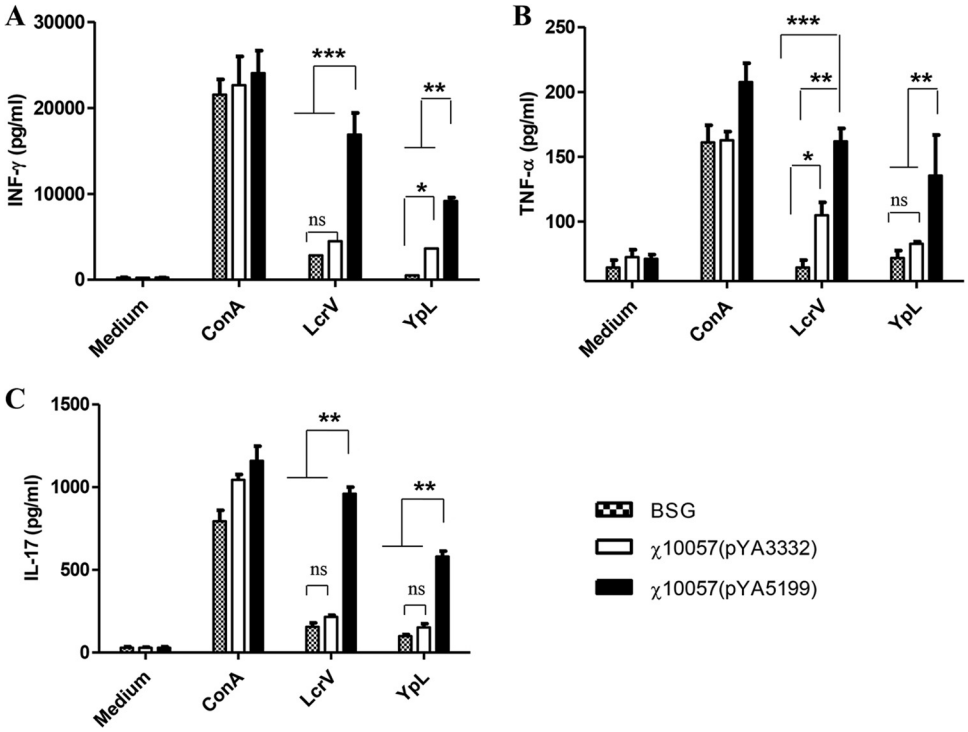

FIG 7 Cellular immune responses in mice immunized with a live attenuated *Y. pseudotuberculosis* strain. Splenocytes isolated from Swiss Webster mice vaccinated orally with strain χ10057(pYA3332) (vector control), χ10057(pYA5199) ($yopE_{Nt138}$-*lcrV*), or BSG at 21 days after initial immunization were stimulated *in vitro* with 4 μg/ml of either purified LcrV or YpL. The mitogen concanavalin A (ConA; 1 μg/ml) served as the positive control, and RPMI 1640 media were used as negative controls. Levels of antigen-specific IFN-γ, TNF-α, and IL-17 cytokines in the culture supernatants produced from splenic cells after 3 days of stimulation were measured by Bioplex assays. (A) Production of IFN-γ. (B) Production of TNF-α. (C) Production of IL-17. Data represent the means ± standard errors (SE) of the results determined for 8 mice for each experiment (the results of two experiments were pooled). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

taneously may improve their immunogenicity to confer protective immunity against pneumonic plague.

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We declare that we have no conflicts of interest.

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