INFECTION AND IMMUNITY, Mar. 2010, p. 1304–1313 Vol. 78, No. 3
0019-9567/10/$12.00 doi:10.1128/IAI.01122-09
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Yersinia pestis with Regulated Delayed Attenuation as a Vaccine Candidate To Induce Protective Immunity against Plague[▽]

Wei Sun, Kenneth L. Roland, Xiaoying Kuang, Christine G. Branger, and Roy Curtiss III*

Center for Infectious Disease and Vaccinology, The Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, Arizona 85287

Received 5 October 2009/Returned for modification 23 November 2009/Accepted 5 January 2010

Two mutant strains of *Yersinia pestis* KIM5+, a Δ*crp* mutant and a mutant with arabinose-dependent regulated delayed-shutoff *crp* expression (*araC* P_{BAD} *crp*), were constructed, characterized *in vitro*, and evaluated for virulence, immunogenicity, and protective efficacy in mice. Both strains were highly attenuated by the subcutaneous (s.c.) route. The 50% lethal doses (LD_{50}s) of the Δ*crp* and *araC* P_{BAD} *crp* mutants were approximately 1,000,000-fold and 10,000-fold higher than those of *Y. pestis* KIM5+, respectively, indicating that both strains were highly attenuated. Mice vaccinated s.c. with 3.8×10^7 CFU of the Δ*crp* mutant developed high anti-*Y. pestis* and anti-LcrV serum IgG titers, both with a strong Th2 bias, and induced protective immunity against subcutaneous challenge with virulent *Y. pestis* (80% survival) but no protection against pulmonary challenge. Mice vaccinated with 3.0×10^4 CFU of the *araC* P_{BAD} *crp* mutant also developed high anti-*Y. pestis* and anti-LcrV serum IgG titers but with a more balanced Th1/Th2 response. This strain induced complete protection against s.c. challenge and partial protection (70% survival) against pulmonary challenge. Our results demonstrate that arabinose-dependent regulated *crp* expression is an effective strategy to attenuate *Y. pestis* while retaining strong immunogenicity, leading to protection against the pneumonic and bubonic forms of plague.

Bubonic and pneumonic plague are zoonotic diseases endemic in many parts of the world, including the United States, and have resulted in over 200 million deaths over the course of human history (51). The etiological agent of plague is *Yersinia pestis*. Although the number of confirmed plague cases that occur worldwide has stabilized over the last 50 years at an average of about 1,700 per year, plague remains a serious public health threat in some regions of the world and outbreaks still occur (19). In addition to the potential for natural infections, *Y. pestis* is generally considered to be among the top five potential biological weapons (19). Recent efforts to create a safe and effective pneumonic plague vaccine have focused on the development of recombinant subunit vaccines that elicit antibodies against two well-characterized *Y. pestis* antigens, the F1 capsule and the virulence protein LcrV (2, 8, 40, 53). A plague vaccine based on live attenuated *Y. pestis* provides the theoretical advantage of simultaneously priming against many antigens, thereby greatly enhancing the likelihood of broad-based protection. In the past, live attenuated strains were generated by selection, rather than precise genetic manipulation, thus raising concern about their genetic composition and stability. The live EV76 vaccine is an apparent *pgm* mutant that has been used in some countries (49). However, a concern is that the EV76 vaccine strain can cause disease in primates, raising questions about its suitability as a human vaccine (29). Nevertheless, as recently as 2002, USAMRIID researchers noted, "Despite their drawbacks, there is ample evidence that live-attenuated strains of *Y. pestis* should be considered as potential vaccine candidates" (52). Research toward the development of new, improved live attenuated vaccines should continue and be strongly encouraged (45). In this work, we explore the feasibility of developing a live attenuated *Y. pestis* vaccine.

The cyclic AMP receptor protein (Crp) is an important transcription factor that regulates the expression of more than 100 genes in *Escherichia coli*, most of which are involved in catabolism of carbon sources other than glucose (24). *E. coli* preferentially utilizes glucose and catabolizes other sugars only when the supply of glucose has become depleted (reviewed in reference 39).

Crp modulates expression of genes in many pathogenic bacteria, including *Salmonella*, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Y. pestis* (10, 38, 44, 54). Crp regulation occurs either directly, by binding to specific DNA sequences near the regulated gene, or indirectly, through the action of CyaR, a regulatory RNA (13). In *Salmonella*, several virulence factors are known to be regulated by *crp*, including *sirA*, which regulates the expression of a number of key invasion genes encoded in *Salmonella* pathogenicity island 1 (SPI1) (1), including *hilA* (20). SPI1 encodes a type 3 secretion system and effector molecules that direct *Salmonella* invasion of mucosal tissues. In addition, *crp* regulates expression of the *spv* genes, also required for invasion of mucosal tissues, and several fimbrial operons (14, 33). Strains of *Salmonella* and *Y. enterocolitica* with *crp* deletions are attenuated in mice and stimulate protective immunity against subsequent challenge with the wild-type parent (10, 38). In *Y. pestis*, the *crp* gene directly regulates expression of some 37 genes, including *ypkA* and *yopO*, which encode a secreted virulence factor, and the laterally acquired plasmid genes *pla*, a known virulence factor, and *pst*, encoding

* Corresponding author. Mailing address: The Biodesign Institute, Center for Infectious Diseases and Vaccinology, Arizona State University, P.O. Box 875401, 1001 S. McAllister Avenue, Tempe, AZ 85287-5401. Phone: (480) 727-0445. Fax: (480) 727-0466. E-mail: rcurtiss@asu.edu.

▽ Published ahead of print on 19 January 2010.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or annotation	Source, reference, or derivation
Strains		
E. coli		
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lac*ZΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
χ6212	*asd*-negative DH5α derivative	31
Y. pestis		
KIM6+	Pgm^+ pMT1 pPCP1, cured of pCD1	16
KIM5+	Pgm^+ pMT1 pPCP1 pCD1Ap	16
KIM5	Pgm^- pMT1 pPCP1 pCD1Ap	5
χ10010	Δ*crp-18 Y. pestis* KIM6+	This study
χ10017	ΔP_{crp21}::TT *araC* P_{BAD} *crp Y. pestis* KIM6+	This study
Plasmids		
pUC18	Ap^r	Invitrogen
pKD46	*repA101*(Ts) *ori* λ Red recombinase expression plasmid	12
pYA3493	Asd^+ pBR *ori* β-lactamase signal sequence-based periplasmic secretion plasmid	21
pYA3700	TT *araC* P_{BAD} cassette plasmid, Ap^r	9
pYA4373	*cat-sacB* cassette in the PstI and SacI sites of pUC18	48
pYA4443	Six-His tag in the C-terminal end of *lcrV* gene cloned into EcoRI and HindIII sites of pYA3493	pYA3493
pYA4579	*y3957′*-*′y3955* fragment ligated by overlapping PCR cloned into EcoRI and HindIII sites of pUC18	pUC18
pYA4581	SD-*crp* and *y3957′* fragments cloned into XhoI/EcoRI sites and PstI/HindIII sites of pYA3700	pYA3700
pYA4588	*cat-sacB* cassette from pYA4373 cloned into PstI site of pYA4581	pYA4581

the bacteriocin pesticin (38, 54). In a recent report, *yopJ*, a secreted effector protein involved in immune suppression, was also shown to be negatively regulated by *crp* (55). Crp is required for expression of the Ysc type 3 secretion system and other virulence factors in *Yersinia*, and functional loss of *crp* diminishes Yop secretion by *Y. enterocolitica* and *Y. pestis* (38, 54). The effect of a *crp* mutation on LcrV secretion has not been reported. A *Y. pestis crp* mutant is attenuated for virulence (54).

An attenuated immunogenic live vaccine must be safe, efficacious, and avirulent and not induce disease symptoms. In addition, it must be able to reach, multiply in, and persist for a while in those lymphoid organs necessary to stimulate a protective immune response. Many well-studied means for attenuating pathogens render strains more susceptible to host defense stresses than wild-type virulent strains and/or impair their ability to effectively colonize host lymphoid tissues. To address these problems, work in our laboratory has led to the development of a regulated delayed attenuation system, applied first to *Salmonella*, in which the vaccine strains display features of the wild-type virulent pathogen at the time of immunization to enable the strain to effectively colonize lymphoid tissues and then become completely attenuated *in vivo* to preclude inducing disease symptoms (11). We have applied this new technology to the *crp* gene in *Y. pestis* by constructing a strain in which *crp* expression is dependent on the presence of arabinose, a sugar that is not present in host tissues (22, 25). Arabinose is provided during *in vitro* growth so that the strain expresses *crp*, making it fully functional to interact with host tissues. Once the strain has invaded host cells, where free arabinose is not available, *crp* is no longer expressed and the strain becomes attenuated. We compared the virulence and immunogenicity of the regulated delayed attenuation strain with those of an isogenic Δ*crp* deletion strain of *Y. pestis*. We found that the arabinose-regulated *crp* mutant was attenuated for virulence and induced greater protective immunity against pneumonic plague than did the Δ*crp* strain.

MATERIALS AND METHODS

Media and reagents. Tryptose blood agar (TBA) and heart infusion broth (HIB) were from Difco. *Y. pestis* strains were grown in HIB and on HIB Congo red agar plates at 30°C to confirm the pigmentation (Pgm) phenotype of *Y. pestis* strains (46). Ampicillin, chloramphenicol (Cm), and L-arabinose were from Sigma (St. Louis, MO). Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England Biolabs. *Taq* DNA polymerase (New England Biolabs) was used in all PCR tests. Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, gel purify fragments, or purify PCR products. T4 ligase, T4 DNA polymerase, and shrimp alkaline phosphatase (SAP) were from Promega.

Bacterial strains and plasmids. Strains and plasmids used in this paper are listed in Table 1. *E. coli* TOP10 was used for plasmid propagation. During screening for mutants, *Y. pestis* was grown on TBA plates with added chloramphenicol (10 μg/ml) or 5% sucrose. *Y. pestis* was grown at 30°C for 24 h with shaking (liquid media) or for 48 h (solid media) (46).

Plasmid construction. All primers used in this paper are listed in Table 2. Primer sets CRP-1/CRP-2 and CRP-3/CRP-4 were used for amplifying the *y3957′* (upstream of *crp*) and *′y3955* (downstream of *crp*) fragments, respectively. Complementarity between primers CRP-2 and CRP-3 is indicated by bold lettering in Table 2. The *′y3955* and *y3957′* fragments were fused by overlapping PCR using primers CRP-1 and CRP-4. The resulting PCR product was digested with EcoRI and HindIII and ligated with pUC18 digested with the same enzymes to construct the plasmid pYA4597.

Primer sets CRP-5/CRP-6 and CRP-7/CRP-8 were used for amplifying the *crp* fragment containing its original Shine-Dalgarno (SD) sequence (SD-*crp*) and the *y3957′* (−110 to −660 bp upstream of *crp*) fragment, respectively. The SD-*crp* and *y3957′* fragments were cloned into the XhoI/EcoRI sites and PstI/HindIII sites of pYA3700, respectively, to form pYA4581. Plasmid pYA4581 was PstI digested, blunted by T4 DNA polymerase, and dephosphorylated with SAP. The *cat-sacB* fragment was cut from pYA4373 using PstI and SacI restriction endonucleases and blunted by T4 DNA polymerase. The two fragments were ligated to form plasmid pYA4588. *lcrV* encoding a C-terminal six-His tag was amplified from pCD1Ap using primers lcrV-1 and lcrV-2 and cloned into the EcoRI and HindIII sites of pYA3493 to form pYA4443.

Preparation of LcrV antiserum. Full-length His-tagged LcrV was expressed from *E. coli* χ6212(pYA4443) and isolated by nickel chromatography. One hundred fifty micrograms of His-tagged LcrV protein was emulsified with Freund's

TABLE 2. Primers used in this study[a]

Name	Sequence
CRP-1	5′ CGGAAGCTTGAGACTGAAAATAGCGGCGA 3′ (HindIII)
CRP-2	5′ **GCGACTGCAGGCTGCCGAGCTCTTC**CCTCTAAAAACCGGCGTTA 3′
CRP-3	5′ **GAAGAGCTCGGCAGCCTGCAGTCGC**TGTTATCCTCTGTTGTTATCG 3′
CRP-4	5′ CGGGAATTCCTTTTTGTAAAATAGACACG 3′ (EcoRI)
CRP-5	5′ CGGGAATTCTTAACGGGTGCCGTAAACGA 3′ (EcoRI)
CRP-6	5′ CGGCTCGAGGAGGATAACAGCGAATGGTT 3′ (XhoI)
CRP-7	5′ CGGCTGCAGGCCGAAAGGTATAGCCAAGGT 3′ (PstI)
CRP-8	5′ CGGAAGCTTCTGATAGATCAACTGCGCGC 3′ (HindIII)
CRP-9	5′ CGACTTCGCGTACCTCAAAGCT 3′
CRP-10	5′ TACATAACCGGAACCACAACCAG 3′
Cm-V	5′ GTTGTCCATATTGGCCACGTTTA 3′
SacB-V	5′ GCAGAAGAGATATTTTTAATTGTGGACG 3′
araC-V	5′ CATCCACCGATGGATAATCGGGTA 3′
lcrV-1	CGGGAATTCATGATTAGAGCCTACGAACA (EcoRI)
lcrV-2	CGGAAGCTTTCAATGATGATGATGATGGTGTTTACCAGACGTGTCATCTAG (HindIII)

[a] Restriction endonuclease sites are underlined. Boldface shows the reverse complementary region between CRP-3 and CRP-4.

complete adjuvant and injected into New Zealand White female rabbits from Charles River Laboratories. The rabbits were immunized with two booster injections (in Freund's incomplete adjuvant) at 3-week intervals. Antiserum was collected 1 week after the last booster injection.

Strain construction. *Y. pestis* mutant strains χ10010 and χ10017 were constructed using the two-step recombination method (48). The procedure was as follows: *Y. pestis* KIM6+(pKD46) was electroporated with the linear *y3957′-cat-sacB*-TT *araC* P_{BAD} SD-*crp* fragment excised from plasmid pYA4588 using EcoRI and HindIII. Electroporants were selected on TBA-chloramphenicol (Cm) plates and verified by PCR. Colonies with the correct PCR profile were streaked onto TBA-Cm-sucrose plates to verify sucrose sensitivity and onto HIB Congo red-Cm plates to confirm the presence of the *pgm* locus. This intermediate strain was used for all further constructions. To construct strain χ10017, the chromosomal *cat-sac* cassette was removed by electroporation with 1 μg of linear DNA (*y3957′*-TT *araC*) cut from pYA4581 using HindIII and BamHI. The loss of the *cat-sac* cassette in sucrose-resistant colonies was confirmed by PCR. Strain χ10010 was constructed by electroporating the intermediate strain with a linear *'y3955-y3957′* fragment cut from pYA4597 using HindIII and EcoRI to delete the entire *crp* gene. Plasmid pKD46 was cured from a single-colony isolate of the above strains to yield χ10010 (Δ*crp*) and χ10017 (*araC* P_{BAD} *crp*). Under biosafety level 3 (BSL-3) containment, plasmid pCD1Ap was then introduced by electroporation into each strain, yielding χ10010(pCD1Ap) and χ10017(pCD1Ap).

SDS-PAGE and immunoblot analyses. Secreted proteins were prepared by using a modification of previously described methods (36). *Y. pestis* was grown in HIB medium overnight at 26°C. Cells were harvested, washed three times in chemically defined medium PMH2 (16), used to inoculate 40 ml of fresh PMH2 medium to an optical density at 600 nm (OD_{600}) of 0.05, and shaken at 26°C overnight. Cultures were shifted to 37°C for 6 h with mild aeration. The OD_{600}s of cultures were measured, and bacterial cell pellets were collected by centrifugation. The pellets were suspended in sodium dodecyl sulfate (SDS) loading buffer. The volume of sample buffer was adjusted based on the OD_{600} to normalize the amount loaded. Cells were lysed by being heated 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). Insoluble materials were removed by centrifugation at 12,500 × *g* for 15 min, and the soluble protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA). 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 (3).

Virulence analysis in mice. All animal procedures were conducted in animal BSL-3 containment facilities and approved by the Arizona State University Animal Care and Use Committee. Single colonies of *Y. pestis* KIM5+ strains to be tested in mice were used to inoculate HIB containing 25 μg/ml ampicillin and grown at 26°C overnight. Bacteria were diluted into 10 ml of fresh medium with 0.2% xylose and 2.5 mM $CaCl_2$ to an OD_{620} of 0.1 and incubated at 26°C for s.c. infections (bubonic plague) or incubated at 37°C for intranasal (i.n.) infections (pneumonic plague) and grown to an OD_{620} of 0.6. The cells were harvested by centrifugation and suspended in 1 ml of isotonic phosphate-buffered saline (PBS).

Female 7-week-old Swiss Webster mice from Charles River Laboratories were inoculated s.c. with 100 μl of the bacterial suspension. Actual numbers of CFU inoculated were determined by plating serial dilutions onto TBA. To determine the 50% lethal dose (LD_{50}), five groups of six mice/group were inoculated i.n. or s.c. with serial dilutions of bacteria. Mice were monitored twice daily for 21 days, and the LD_{50} was calculated as described previously (41). For *in vivo* complementation of strain χ10017(pCD1Ap), 120 mg of L-arabinose dissolved in 100 μl PBS was intraperitoneally administered to mice on the day of inoculation and once a day thereafter (28).

For colonization/dissemination analysis, groups of mice were injected s.c. At the indicated times after infection, 3 mice per strain were euthanized, and samples of blood, lungs, spleen, and liver were removed. The bacterial load for each organ was determined by plating dilutions of the homogenized tissues onto TBA plates containing 25 μg/ml ampicillin and reported as CFU per gram of tissue or CFU per ml blood. Infections were performed in at least two independent experiments.

Determination of protective efficacy. *Y. pestis* strains were grown as described above. Two groups of Swiss Webster mice (10/group) were vaccinated s.c. with 3.8×10^7 CFU of χ10010(pCD1Ap) or 3×10^4 CFU of χ10017(pCD1Ap) cells in 100 μl of PBS on day 0. Another two groups of mice (4/group) were injected with 100 μl of PBS as controls. Blood was collected by retro-orbital sinus puncture at 2 and 4 weeks postimmunization and 2 weeks after challenge for antibody measurement. Mice were lightly anesthetized using a ketamine and xylazine mixture administered intramuscularly before bleeding. On day 35, animals were challenged s.c. with *Y. pestis* KIM5+ at either 1.3×10^5 CFU for the χ10017(pCD1Ap) group or 1.3×10^7 CFU for the χ10010(pCD1Ap) group in 100 μl PBS or lightly anesthetized with a 1:5 xylazine-ketamine mixture and challenged i.n. with 1.4×10^4 CFU in 20 μl PBS. Control groups were challenged with 1.3×10^3 CFU by both routes. All infected animals were observed over a 15-day period for the development of signs of plague infection.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was used to assay serum IgG antibodies against *Yersinia* whole-cell lysates (YpL) (47) and purified LcrV antigen of *Y. pestis* KIM5+. Polystyrene 96-well flat-bottomed microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 200 ng/well of YpL or purified LcrV protein. The procedures were same as those described previously (21).

Measurement of cytokine concentrations. Cytokines were quantitated by a double-sandwich ELISA as described previously (42). Mice in groups of three were euthanized at intervals by terminal bleeding under anesthesia. Pooled blood was allowed to clot overnight at 4°C, and serum was separated by centrifugation at 10,000 × *g* for 10 min. Sera were filtered once through a 0.22-μm syringe filter, cultured on TBA to confirm that bacteria had been removed, and stored at −70°C prior to assay.

Commercial solid-phase enzyme immunoassays utilizing the multiple-antibody sandwich principle were used to determine cytokines in biological samples. Levels of interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) were determined with mouse IL-10, IFN-γ, and TNF-α Ready-Set-Go kits (eBioscience), respectively. Concentrations of cytokines were measured by reading optical density at 450 nm and then calculated in reference to values obtained in standard curves generated for each assay. Assays of pooled sera were repeated three times.

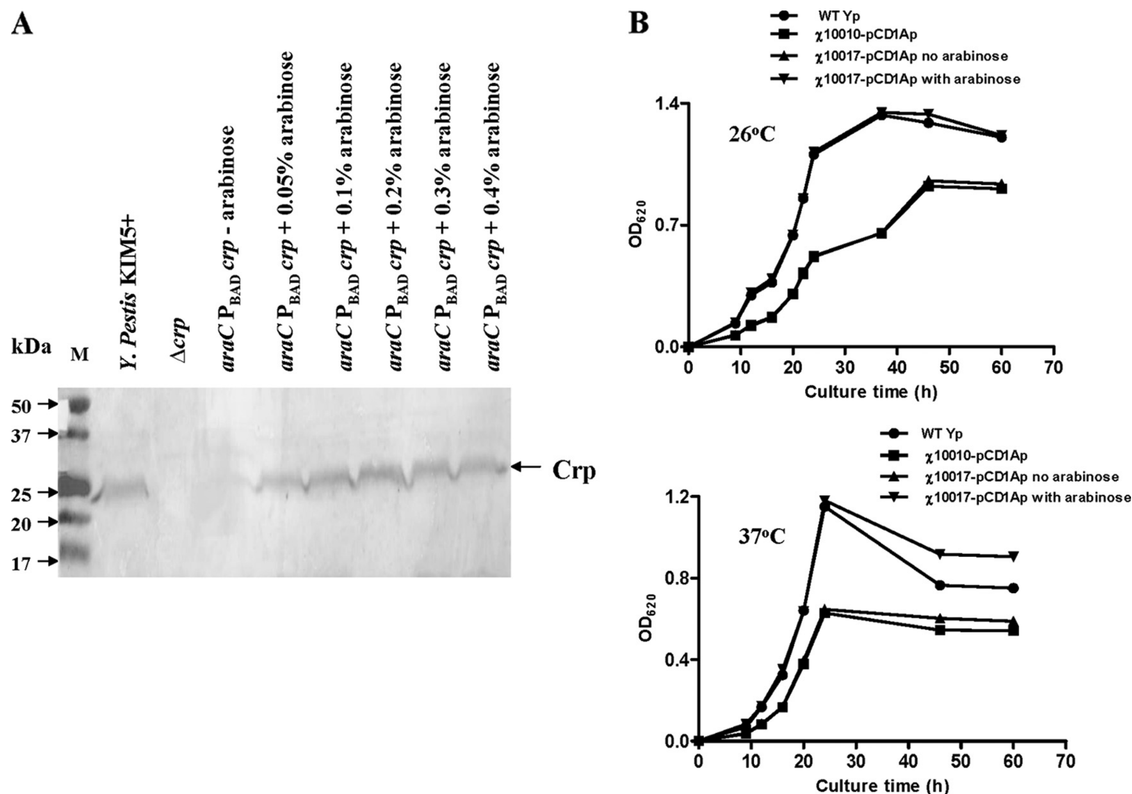

FIG. 1. Crp synthesis and growth of *Y. pestis* mutants. (A) Measurement of Crp synthesis in *Y. pestis* KIM5+, χ10010 (*crp18*), and χ10017 (*araC* P_{BAD} *crp*). Strains were grown in HIB at 37°C overnight, and Crp synthesis was detected by Western blotting using anti-Crp sera. M, protein marker. (B) Growth of *Y. pestis* strains in HIB medium at 26°C or 37°C. ●, *Y. pestis* KIM5+; ■, χ10010(pCD1Ap) (Δ*crp*); ▲, χ10017(pCD1Ap) (*araC* P_{BAD} *crp*) without arabinose; ▼, χ10017(pCD1Ap) with 0.05% arabinose. WT, wild type.

Statistical analysis. The log rank test was used for analysis of the survival curves. Data are expressed as means ± standard errors (SE). The Student *t* test was used for other statistical analyses. A *P* value of <0.05 was considered significant.

RESULTS

Crp synthesis and growth of *Y. pestis* mutants. We constructed mutant *Y. pestis* strains χ10010 (Δ*crp*) and χ10017 (*araC* P_{BAD} *crp*). In the *araC* P_{BAD} *crp* mutant χ10017, *crp* expression is dependent on the presence of arabinose. Crp was not detected in either the Δ*crp* strain χ10010 or the *araC* P_{BAD} *crp* strain χ10017 grown in the absence of arabinose (Fig. 1A). Upon arabinose addition, χ10017 synthesized roughly the same amount of Crp as did wild-type *Y. pestis*.

Once we had confirmed that Crp synthesis was arabinose regulated, we moved plasmid pCD1Ap into both mutants and examined their growth in liquid media. Strains χ10010 (pCD1Ap) and χ10017(pCD1Ap) without arabinose grew more slowly and did not reach the same final OD_{620} as did *Y. pestis* KIM5+ at 26°C or 37°C in HIB medium (Fig. 1B). When 0.05% arabinose was included in the growth medium, χ10017(pCD1Ap) grew at the same rate as did the wild type.

LcrV synthesis and secretion in *Y. pestis* KIM5+ and mutant derivatives. Crp is required for expression of the Ysc type 3 secretion system and other virulence factors in *Yersinia*, and functional loss of *crp* diminishes Yop secretion by *Y. enterocolitica* and *Y. pestis* (7, 23, 38). However, the effect of a *crp* mutation on LcrV secretion has not been reported. Therefore, we compared LcrV production in cells and supernatants from *Y. pestis* KIM5+, χ10010(pCD1Ap), and χ10017(pCD1Ap). We observed no difference in LcrV synthesis in whole-cell lysates among strains (Fig. 2). There was a reduction in the amount of LcrV detected in supernatants between the wild type and strains χ10010(pCD1Ap) (Δ*crp*) and χ10017 (pCD1Ap) (*araC* P_{BAD} *crp*). Wild-type levels of secreted LcrV were restored when strain χ10017(pCD1Ap) was grown with 0.05% arabinose (Fig. 2).

Virulence of *Y. pestis* mutants in mice. To investigate the contribution of Crp to *Y. pestis* virulence, we infected Swiss Webster mice s.c. with *Y. pestis* KIM5+, χ10010(pCD1Ap) (Δ*crp*), or χ10017(pCD1Ap) (*araC* P_{BAD} *crp*). Strain χ10017(pCD1Ap) was grown in the presence of arabinose prior to inoculation. The LD_{50} of *Y. pestis* KIM5+ was <10 CFU, consistent with previous results (50). The LD_{50} of the Δ*crp* mutant χ10010(pCD1Ap) was $>3 \times 10^7$ CFU. The LD_{50} of strain χ10017(pCD1Ap) was 4.3×10^5 CFU, and the mean time to death was delayed 2 to 9 days compared to the wild type. The LD_{50} of χ10017(pCD1Ap) was the same as that of KIM5+ (LD_{50}, <10 CFU) when inoculated mice were injected with arabinose, indicating full complementation of the attenuation phenotype. In a preliminary experiment, we found that both the Δ*crp* and *araC* P_{BAD} *crp* mutants were attenuated when administered by the intranasal route, with LD_{50}s of $>1 \times 10^4$ CFU. However, mice inoculated with 7×10^3 to 9×10^3

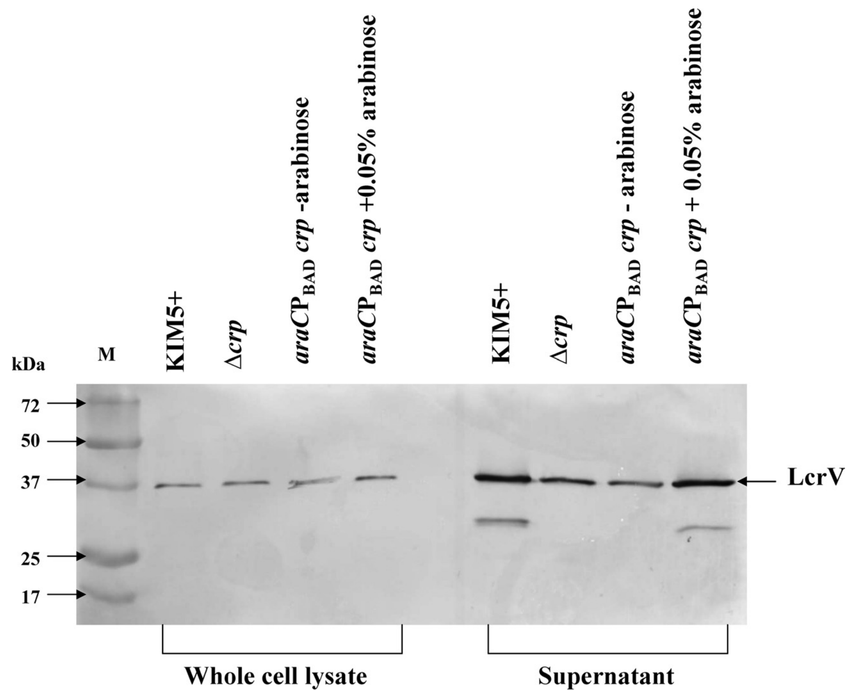

FIG. 2. Measurement of LcrV synthesis and secretion in *Y. pestis* by Western blot analysis. Whole-cell lysates and supernatant fractions were separated by SDS-PAGE and detected by Western blotting. For each sample, equivalent amounts of protein were loaded. The *araC* P_{BAD} *crp* strain χ10017(pCD1Ap) was grown with and without 0.05% arabinose.

CFU of either mutant were not protected from subsequent intranasal challenge with 5×10^3 CFU of KIM5+ (data not shown) and, therefore, we did not repeat those experiments.

We evaluated the ability of the *Y. pestis* mutants to disseminate systemically compared to *Y. pestis* KIM5+ by monitoring, over a 9-day period, the lungs, spleen, liver, and blood of groups of mice injected with each of the strains. Because of the difference in LD_{50}s among the three strains, we inoculated mice with different doses of each. For this type of experiment, we typically choose a dose that is higher than the LD_{50}. However, since we were not able to establish an LD_{50} value for the Δ*crp* strain ($>1 \times 10^7$ CFU), we chose a dose that matched the highest dose for which we had data. For the *araC* P_{BAD} *crp* mutant, we chose a dose that was 10-fold above the LD_{50}. Thus, mice were inoculated with 1.5×10^3 CFU of *Y. pestis* KIM5+, 4.2×10^7 CFU of χ10010(pCD1Ap), or 3.8×10^6 CFU of χ10017(pCD1Ap). The kinetics of infection were similar for the two mutants. At 3 days postinfection (p.i.), the numbers of bacteria recovered from the blood, liver, and spleen were similar for all strains (Fig. 3). About half as many χ10010 and χ10017 cells as those of the wild-type strain were recovered from lungs. The numbers of mutants recovered from all tissues decreased steadily on days 6 and 9. All mice inoculated with *Y. pestis* KIM5+ succumbed to the infection before day 9, and therefore, we do not include any of those mice in our figure for that time point.

Evaluation of protective immunity. Groups of mice were immunized with a single dose of χ10010(pCD1Ap) (Δ*crp*), 10017(pCD1Ap) (*araC* P_{BAD} *crp*), or *Y. pestis* KIM5 (Pgm^-) and challenged 35 days later. For these experiments, we wanted to use the highest possible immunizing dose for each strain. We based our decision on immunizing doses for each strain on the LD_{50} data, shown above. Therefore, we immunized with a dose of 3×10^4 CFU of 10017(pCD1Ap), 3.8×10^7 CFU of 10010(pCD1Ap), or 2.5×10^7 CFU of *Y. pestis* KIM5 (Pgm^-), respectively. Our results after challenge show that a single s.c. dose of χ10010(pCD1Ap) or *Y. pestis* KIM5 (Pgm^-) provided excellent protection against a 1×10^6-LD_{50} s.c. challenge (Fig. 4A). A single s.c. dose of χ10017(pCD1Ap) provided complete protection against a 10,000-LD_{50} s.c. challenge without any symptoms (Fig. 4B). Immunization with strain χ10010(pCD1Ap) delayed the time of death but ultimately did not provide protection against a 100-LD_{50} i.n. challenge. Immunization with the *Y. pestis* strain χ10017(pCD1Ap) or the *pgm* mutant strain KIM5 provided significant protection ($P < 0.001$), protecting most of the mice against a 100-LD_{50} i.n. challenge (Fig. 4C). None of the mice immunized with PBS survived challenge by either route (Fig. 4).

Serum immune responses. Serum IgG responses to YpL and LcrV from immunized mice were measured by ELISA. High anti-YpL (Fig. 5A) titers were slower to develop for the arabinose-regulated *crp* mutant, χ10017(pCD1Ap), than for the Δ*crp* mutant, χ10010(pCD1Ap), but by week 4, the titers were similar. Also by week 4, the anti-LcrV (Fig. 5B) serum IgG titers were somewhat higher in mice immunized with χ10017(pCD1Ap) than in mice immunized with χ10010 (pCD1Ap). Titers against both antigens were boosted in mice challenged s.c. No boosting was observed in the mice immunized with χ10017(pCD1Ap) after i.n. challenge.

Th1 cells direct cell-mediated immunity and promote class switching to IgG2a, and Th2 cells provide potent help for B-cell antibody production and promote class switching to

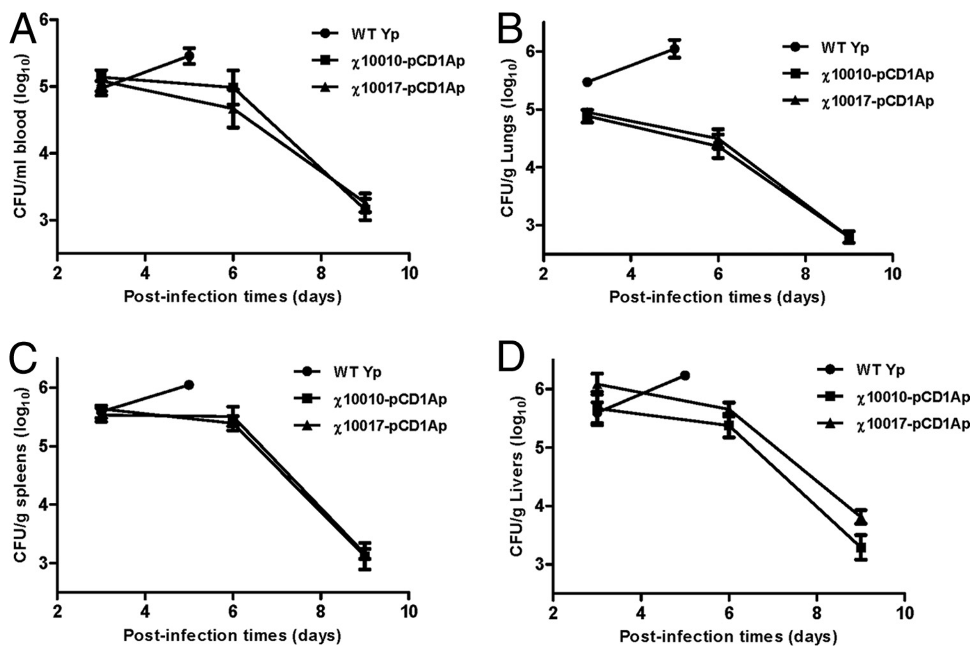

FIG. 3. Kinetics of infection with *Y. pestis* KIM5+ and mutant derivatives in mouse tissues. Bacteria were inoculated s.c. with 1.5×10^3 CFU of *Y. pestis* KIM5+, 4.2×10^7 CFU of χ10010(pCD1Ap), or 3.8×10^6 CFU of χ10017(pCD1Ap), and at various times CFU per organ in the blood (A), lungs (B), spleens (C), and livers (D) were determined. Error bars represent standard deviations. We examined 3 mice/group/time point, and the experiment was performed twice with similar results. WT, wild type.

IgG1 (17). The Δ*crp* strain χ10010(pCD1Ap) elicited a strong Th2-biased response against both antigens, with high IgG1 titers and low IgG2 titers (Fig. 6A and B). Strain χ10017 (pCD1Ap) induced a more balanced Th1/Th2 response (Fig. 6C and D). Challenge did not have much effect on the IgG1/IgG2a ratios, except for the anti-LcrV response in mice immunized with χ10010(pCD1Ap), where the response became more balanced.

Induction of cytokines. Cytokines are critical to the development and functioning of both the innate and adaptive immune responses. They are secreted by immune cells that have encountered pathogens, thereby activating and recruiting additional immune cells to respond to the infection. LcrV is an immunomodulator, inhibiting production of TNF-α and IFN-γ and inducing IL-10 in eukaryotic cells both *in vivo* and *in vitro* (4, 30, 32). To evaluate the effect of reduced LcrV secretion in the two mutants (Fig. 2), we compared production of IL-10, IFN-γ, and TNF-α in infected mice. Groups of three Swiss-Webster mice were inoculated s.c. with 1,500 CFU of *Y. pestis* KIM5+, 4.2×10^7 CFU of χ10010(pCD1Ap), or 3.8×10^6 CFU of χ10017(pCD1Ap). A group of uninfected mice served as controls. Blood was collected via cardiac puncture at days 3 and 6 p.i. for cytokine analysis. We could detect IL-10 but not IFN-γ or TNF-α in the sera of animals infected with *Y. pestis* KIM5+, but IL-10 and proinflammatory factors such as IFN-γ and TNF-α were not detected in mice infected with χ10010(pCD1Ap) and χ10017(pCD1Ap) (data not shown).

DISCUSSION

We have been developing live bacterial vaccine strains that display regulated delayed attenuation, such that upon immunization, the vaccine strain exhibits all the phenotypic qualities of the wild type, allowing it to establish an infection necessary to stimulate an immune response. After 5 to 10 rounds of replication, the strain becomes attenuated, precluding its ability to cause disease. We have shown this in detail previously for *Salmonella* (11), and here we apply this concept to *Y. pestis*. With this goal in mind, we constructed and evaluated an *araC* P_{BAD} *crp* strain and, for comparison, an isogenic Δ*crp* strain. Our results indicate that *crp* affects virulence in *Y. pestis*, consistent with what was found in a recent study in which a *Y. pestis* Δ*crp* strain was shown to be highly attenuated when administered subcutaneously to mice (54). In that study, the authors found that the s.c. LD_{50} in mice was approximately 1×10^4-fold higher than that of wild-type *Y. pestis* strain 201 (54). The Δ*crp* mutant used in our study was completely avirulent, with an $LD_{50} > 1 \times 10^6$-fold greater than that of the wild type. Possible reasons for this difference include the use of different mouse strains (BALB/c versus Swiss Webster), the use of different parental strains, and differences in the way that the *crp* mutant was constructed (deletion versus insertion). These discrepancies aside, our work confirms that a *Y. pestis crp* mutant is highly attenuated when administered subcutaneously.

The increased LD_{50} observed for both *crp* mutants by s.c. and i.n. routes indicates that Crp regulates genes important in establishing a lethal infection during bubonic and pneumonic plague. A number of factors may play a role in the loss of virulence. In the absence of *crp* expression, there is a reduction in the *in vitro* growth rate as observed by Zhan et al. (54). The *pla* gene, important for systemic spread of *Y. pestis* from subcutaneous sites, requires Crp for maximal expression (23, 54). Pla is also involved in the development

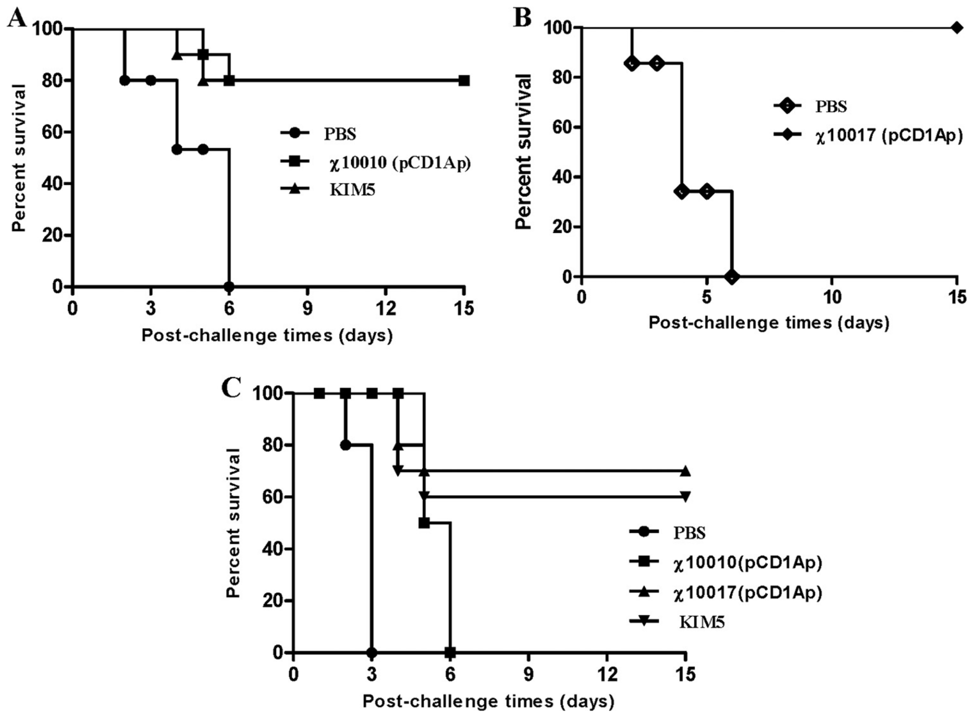

FIG. 4. Survival of immunized and nonimmunized mice after *Y. pestis* KIM5+ challenge. (A) Swiss Webster mice vaccinated s.c. with 3.8×10^7 CFU of χ10010(pCD1Ap) or 2.5×10^7 CFU of *Y. pestis* KIM5 (Pgm$^-$) were challenged with 1.3×10^7 CFU of *Y. pestis* KIM5+ via the s.c. route. (B) Swiss Webster mice vaccinated s.c. with 3.0×10^4 CFU of χ10017(pCD1Ap) were challenged with 1.4×10^5 CFU of *Y. pestis* KIM5+ via the s.c. route. (C) Swiss Webster mice vaccinated s.c. with 3.8×10^7 CFU of χ10010(pCD1Ap), 3.0×10^4 CFU of χ10017(pCD1Ap), or 2.5×10^7 CFU of KIM5 were challenged via the i.n. route with 1.4×10^4 CFU of *Y. pestis* KIM5+. For panels A and B, survival of immunized mice was significantly greater than that for PBS controls in all experiments ($P < 0.001$). For panel C, survival of mice immunized with χ10017(pCD1Ap) or KIM5 was significantly greater than that of mice immunized with strain χ10010(pCD1Ap) or PBS controls ($P < 0.001$). There were 10 mice per vaccination group and 4 mice per control group for each experiment. The experiment was performed twice.

of pneumonic plague (27), which may explain why we observed lower titers of the two mutants than of the wild type in lung tissue on day 3.

Yop secretion is reduced in *crp* mutants (23, 38). The Yop virulon comprises both the Yop effector proteins and the proteins necessary for injecting them into host cells. The injected Yops perturb cytoskeleton dynamics, disrupt phagocytosis, and block the production of proinflammatory cytokines, thus favoring the survival of the invading *Yersinia* (6, 7, 37). Our results show that LcrV secretion was also reduced (Fig. 2). Although controversial, some reports indicate that LcrV stimulates the release of IL-10 through interactions with Toll-like receptor 2 and CD14 receptors, which downregulate synthesis of the proinflammatory cytokines IFN-γ and TNF-α (4, 43). Thus,

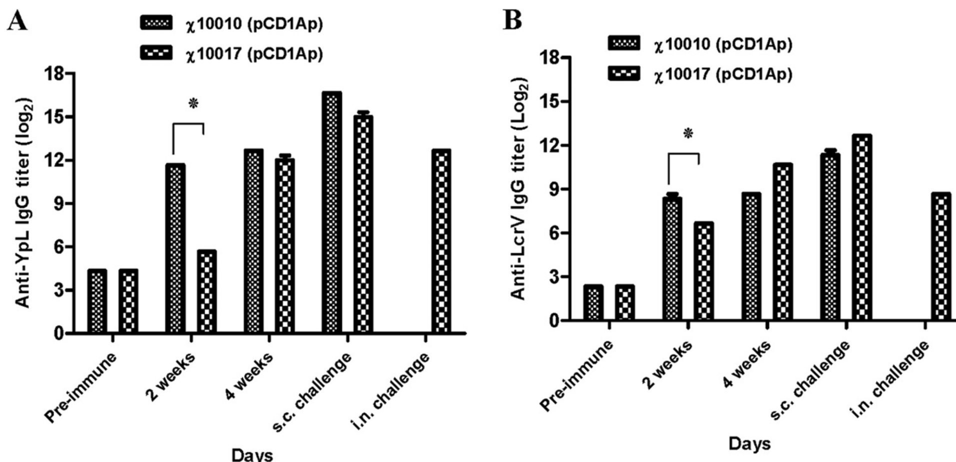

FIG. 5. The IgG response in sera of mice inoculated with χ10010(pCD1Ap) or χ10017(pCD1Ap). (A) *Y. pestis* KIM5+ whole-cell lysate (YpL) was used as the coating antigen. (B) Recombinant LcrV was used as the coating antigen. *, $P < 0.01$.

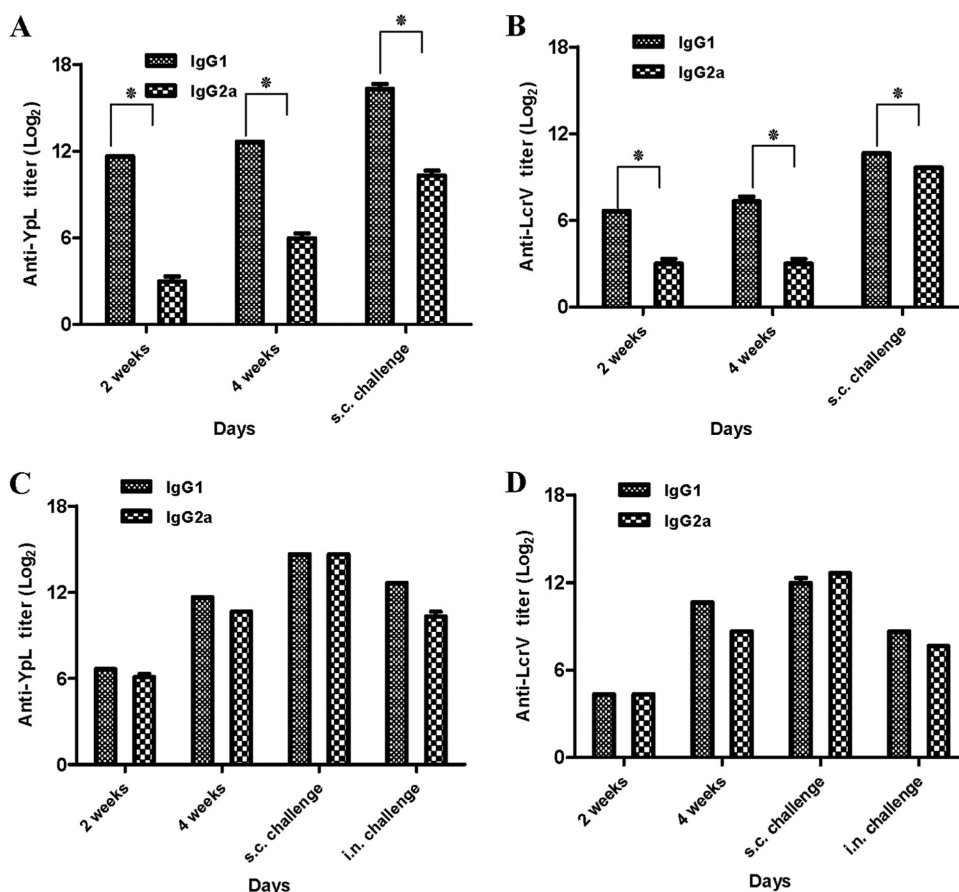

FIG. 6. Serum IgG1 and IgG2a responses to YpL and recombinant LcrV. (A) IgG1 and IgG2a antibody levels to YpL in sera of mice immunized s.c. with χ10010(pCD1Ap). (B) IgG1 and IgG2a antibody levels to recombinant LcrV in sera of mice subcutaneously immunized with χ10010(pCD1Ap). (C) IgG1 and IgG2a antibody levels to YpL in sera of mice subcutaneously immunized with χ10017(pCD1Ap). (D) IgG1 and IgG2a antibody levels to recombinant LcrV in sera of mice subcutaneously immunized with χ10017(pCD1Ap). *, $P < 0.01$.

the reduction of Yop and LcrV secretion may play a role in the avirulence of *crp* mutants.

It has been suggested that *crp* regulates expression of *caf1*, which encodes the F1 protein that forms a capsule on *Y. pestis* at 37°C (54). However, since *caf1* mutants ($F1^-$) are fully virulent in mice and nonhuman primates (15), we focused our attention on the expression of antigens such as Yops and LcrV that will provide protection against all *Y. pestis* strains.

Strain χ10010(pCD1Ap) (Δ*crp*) induced a strong Th2 bias, while strain χ10017(pCD1Ap), featuring regulated *crp* expression, elicited a more balanced Th1/Th2 response (Fig. 6). Both mutants examined in this study provided protection against s.c. challenge (Fig. 4). However, only strain χ10017(pCD1Ap) provided significant protection against pneumonic challenge (Fig. 4C), similar to the *pgm* mutant strain KIM5. These results are consistent with what is known about the immunogenicity requirements for protection against *Y. pestis*. Protection against bubonic plague has been correlated with antibody production (26, 45), while protection against pneumonic plague is known to require cell-mediated immunity induced by IFN-γ and TNF-α (34, 35). The strong Th2 response generated by the Δ*crp* strain leads to a primarily antibody-based response with little priming of cellular immunity, while the more balanced Th1/Th2 response generated by χ10017(pCD1Ap) should provide both antibody and cellular priming (17). The fact that we did not detect IL-10, IFN-γ, and TNF-α in the sera of mice immunized with χ10017(pCD1Ap) may be a reflection of the low sensitivity of the assay that we used (18). Our results support the idea that regulated delayed attenuation is a powerful new tool for designing live *Yersinia* vaccines to stimulate both humoral and cellular immunity.

One of the strategies that we have adopted as for developing live attenuated *Salmonella* vaccine strains has been to include multiple attenuating mutations as a way to ensure an acceptable degree of safety. As we move forward toward developing a live *Y. pestis* vaccine, we will use a similar approach. In future studies, we plan to construct *Y. pestis* vaccine strains with multiple attenuating mutations and evaluate them for immunogenicity, pathological effects on host tissues, and protective efficacy.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health grant AI057885.

All authors declare no conflict of interest.

REFERENCES

1. **Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis, and F. Heffron.** 1999. *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. Mol. Microbiol. **31:**971–982.
2. **Alvarez, M. L., H. L. Pinyerd, J. D. Crisantes, M. M. Rigano, J. Pinkhasov, A. M. Walmsley, H. S. Mason, and G. A. Cardineau.** 2006. Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice. Vaccine **24:**2477–2490.
3. **Branger, C. G., J. D. Fetherston, R. D. Perry, and R. Curtiss III.** 2007. Oral vaccination with different antigens from *Yersinia pestis* KIM delivered by live attenuated *Salmonella* typhimurium elicits a protective immune response against plague. Adv. Exp. Med. Biol. **603:**387–399.
4. **Brubaker, R. R.** 2003. Interleukin-10 and inhibition of innate immunity to *Yersiniae*: roles of Yops and LcrV (V antigen). Infect. Immun. **71:**3673–3681.
5. **Brubaker, R. R.** 1983. The Vwa+ virulence factor of *Yersiniae*: the molecular basis of the attendant nutritional requirement for Ca^{2+}. Rev. Infect. Dis. **5**(Suppl. 4)**:**S748–S758.
6. **Cornelis, G. R.** 2002. *Yersinia* type III secretion: send in the effectors. J. Cell Biol. **158:**401–408.
7. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier.** 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol. Mol. Biol. Rev. **62:**1315–1352.
8. **Cornelius, C. A., L. E. Quenee, K. A. Overheim, F. Koster, T. L. Brasel, D. Elli, N. A. Ciletti, and O. Schneewind.** 2008. Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague. Infect. Immun. **76:**5588–5597.
9. **Curtiss, R., III, and W. Kong.** August 2006. Regulated bacterial lysis for gene vaccine vector delivery and antigen release. U.S. patent 2006/0140975.
10. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella* typhimurium deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55:**3035–3043.
11. **Curtiss, R., III, S. Y. Wanda, B. M. Gunn, X. Zhang, S. A. Tinge, V. Ananthnarayan, H. Mo, S. Wang, and W. Kong.** 2009. *Salmonella enterica* serovar typhimurium strains with regulated delayed attenuation in vivo. Infect. Immun. **77:**1071–1082.
12. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
13. **De Lay, N., and S. Gottesman.** 2009. The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. J. Bacteriol. **191:**461–476.
14. **Eisenstein, B. I., E. H. Beachey, and S. S. Solomon.** 1981. Divergent effects of cyclic adenosine 3′,5′-monophosphate on formation of type 1 fimbriae in different K-12 strains of *Escherichia coli*. J. Bacteriol. **145:**620–623.
15. **Friedlander, A. M., S. L. Welkos, P. L. Worsham, G. P. Andrews, D. G. Heath, G. W. Anderson, Jr., M. L. Pitt, J. Estep, and K. Davis.** 1995. Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. Clin. Infect. Dis. **21**(Suppl. 2)**:**S178–S181.
16. **Gong, S., S. W. Bearden, V. A. Geoffroy, J. D. Fetherston, and R. D. Perry.** 2001. Characterization of the *Yersinia pestis* Yfu ABC inorganic iron transport system. Infect. Immun. **69:**2829–2837.
17. **Gor, D. O., N. R. Rose, and N. S. Greenspan.** 2003. TH1-TH2: a procrustean paradigm. Nat. Immunol. **4:**503–505.
18. **Hillyer, L. M., and B. Woodward.** 2003. Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood. Am. J. Physiol. Regul. Integr. Comp. Physiol. **285:**R1514–R1519.
19. **Inglesby, T. V., D. T. Dennis, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, J. F. Koerner, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, M. Schoch-Spana, and K. Tonat.** 2000. Plague as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. JAMA **283:**2281–2290.
20. **Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller.** 1996. Transcriptional activation of *Salmonella* typhimurium invasion genes by a member of the phosphorylated response-regulator superfamily. Mol. Microbiol. **22:**715–727.
21. **Kang, H. Y., J. Srinivasan, and R. Curtiss III.** 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar typhimurium vaccine. Infect. Immun. **70:**1739–1749.
22. **Katzman, R. L., E. Lisowska, and R. W. Jeanloz.** 1970. Invertebrate connective tissue. Isolation of D-arabinose from sponge acidic polysaccharide. Biochem. J. **119:**17–19.
23. **Kim, T. J., S. Chauhan, V. L. Motin, E. B. Goh, M. M. Igo, and G. M. Young.** 2007. Direct transcriptional control of the plasminogen activator gene of *Yersinia pestis* by the cyclic AMP receptor protein. J. Bacteriol. **189:**8890–8900.
24. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749–795.
25. **Kong, W., S. Y. Wanda, X. Zhang, W. Bollen, S. A. Tinge, K. L. Roland, and R. Curtiss III.** 2008. Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment. Proc. Natl. Acad. Sci. U. S. A. **105:**9361–9366.
26. **Kummer, L. W., F. M. Szaba, M. A. Parent, J. J. Adamovicz, J. Hill, L. L. Johnson, and S. T. Smiley.** 2008. Antibodies and cytokines independently protect against pneumonic plague. Vaccine **26:**6901–6907.
27. **Lathem, W. W., P. A. Price, V. L. Miller, and W. E. Goldman.** 2007. A plasminogen-activating protease specifically controls the development of primary pneumonic plague. Science **315:**509–513.
28. **Loessner, H., A. Endmann, S. Leschner, K. Westphal, M. Rohde, T. Miloud, G. Hammerling, K. Neuhaus, and S. Weiss.** 2007. Remote control of tumour-targeted *Salmonella enterica* serovar Typhimurium by the use of L-arabinose as inducer of bacterial gene expression in vivo. Cell. Microbiol. **9:**1529–1537.
29. **Meyer, K. F., G. Smith, L. Foster, M. Brookman, and M. Sung.** 1974. Live, attenuated *Yersinia pestis* vaccine: virulent in nonhuman primates, harmless to guinea pigs. J. Infect. Dis. **129**(Suppl.)**:**S85–S120.
30. **Motin, V. L., R. Nakajima, G. B. Smirnov, and R. R. Brubaker.** 1994. Passive immunity to *Yersiniae* mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. Infect. Immun. **62:**4192–4201.
31. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an *asd*$^{+}$ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Biotechnology (NY) **6:**693–697.
32. **Nedialkov, Y. A., V. L. Motin, and R. R. Brubaker.** 1997. Resistance to lipopolysaccharide mediated by the *Yersinia pestis* V antigen-polyhistidine fusion peptide: amplification of interleukin-10. Infect. Immun. **65:**1196–1203.
33. **O'Byrne, C. P., and C. J. Dorman.** 1994. The *spv* virulence operon of *Salmonella* typhimurium LT2 is regulated negatively by the cyclic AMP (cAMP)-cAMP receptor protein system. J. Bacteriol. **176:**905–912.
34. **Parent, M. A., K. N. Berggren, L. W. Kummer, L. B. Wilhelm, F. M. Szaba, I. K. Mullarky, and S. T. Smiley.** 2005. Cell-mediated protection against pulmonary *Yersinia pestis* infection. Infect. Immun. **73:**7304–7310.
35. **Parent, M. A., L. B. Wilhelm, L. W. Kummer, F. M. Szaba, I. K. Mullarky, and S. T. Smiley.** 2006. 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. **74:**3381–3386.
36. **Perry, R. D., A. G. Bobrov, O. Kirillina, H. A. Jones, L. Pedersen, J. Abney, and J. D. Fetherston.** 2004. Temperature regulation of the hemin storage (Hms+) phenotype of *Yersinia pestis* is posttranscriptional. J. Bacteriol. **186:**1638–1647.
37. **Perry, R. D., S. C. Straley, J. D. Fetherston, D. J. Rose, J. Gregor, and F. R. Blattner.** 1998. DNA sequencing and analysis of the low-Ca^{2+}-response plasmid pCD1 of *Yersinia pestis* KIM5. Infect. Immun. **66:**4611–4623.
38. **Petersen, S., and G. M. Young.** 2002. Essential role for cyclic AMP and its receptor protein in *Yersinia enterocolitica* virulence. Infect. Immun. **70:**3665–3672.
39. **Postma, P.** 1986. Catabolite repression and related phenomena. Symp. Soc. Gen. Microbiol. **39:**21–49.
40. **Powell, B. S., G. P. Andrews, J. T. Enama, S. Jendrek, C. Bolt, P. Worsham, J. K. Pullen, W. Ribot, H. Hines, L. Smith, D. G. Heath, and J. J. Adamovicz.** 2005. Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. Biotechnol. Prog. **21:**1490–1510.
41. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
42. **Sheehan, K. C., N. H. Ruddle, and R. D. Schreiber.** 1989. Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. J. Immunol. **142:**3884–3893.
43. **Sing, A., D. Rost, N. Tvardovskaia, A. Roggenkamp, A. Wiedemann, C. J. Kirschning, M. Aepfelbacher, and J. Heesemann.** 2002. *Yersinia* V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J. Exp. Med. **196:**1017–1024.
44. **Skorupski, K., and R. K. Taylor.** 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. Proc. Natl. Acad. Sci. U. S. A. **94:**265–270.
45. **Smiley, S. T.** 2008. Current challenges in the development of vaccines for pneumonic plague. Expert Rev. Vaccines **7:**209–221.
46. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51:**445–454.
47. **Sun, W., K. L. Roland, C. G. Branger, X. Kuang, and R. Curtiss III.** 2009. The role of *relA* and *spoT* in *Yersinia pestis* KIM5+ pathogenicity. PLoS One **4:**e6720.
48. **Sun, W., S. Wang, and R. Curtiss III.** 2008. Highly efficient method for introducing successive multiple scarless gene deletions and markerless gene insertions into the *Yersinia pestis* chromosome. Appl. Environ. Microbiol. **74:**4241–4245.
49. **Titball, R. W., and E. D. Williamson.** 2004. *Yersinia pestis* (plague) vaccines. Expert Opin. Biol. Ther. **4:**965–973.

50. **Une, T., and R. R. Brubaker.** 1984. In vivo comparison of avirulent Vwa- and Pgm- or Pstr phenotypes of *Yersiniae*. Infect. Immun. **43:**895–900.
51. **Weekly Epidemiological Record.** 2003. Human plague in 2000 and 2001. Wkly. Epidemiol. Rec. **78:**130–135.
52. **Welkos, S., M. L. Pitt, M. Martinez, A. Friedlander, P. Vogel, and R. Tammariello.** 2002. Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of *Yersinia pestis* in non-human primate and mouse models of pneumonic plague. Vaccine **20:**2206–2214.
53. **Williamson, E. D., H. C. Flick-Smith, E. Waters, J. Miller, I. Hodgson, C. S. Le Butt, and J. Hill.** 2007. Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates. Microb. Pathog. **42:**11–21.
54. **Zhan, L., Y. Han, L. Yang, J. Geng, Y. Li, H. Gao, Z. Guo, W. Fan, G. Li, L. Zhang, C. Qin, D. Zhou, and R. Yang.** 2008. The cyclic AMP receptor protein, CRP, is required for both virulence and expression of the minimal CRP regulon in *Yersinia pestis* biovar microtus. Infect. Immun. **76:**5028–5037.
55. **Zhan, L., L. Yang, L. Zhou, Y. Li, H. Gao, Z. Guo, L. Zhang, C. Qin, D. Zhou, and R. Yang.** 2009. Direct and negative regulation of the *sycO-ypkA-ypoJ* operon by cyclic AMP receptor protein (CRP) in *Yersinia pestis*. BMC Microbiol. **9:**178.

Editor: J. B. Bliska