Evaluation of Protective Potential of *Yersinia pestis* Outer Membrane Protein Antigens as Possible Candidates for a New-Generation Recombinant Plague Vaccine

Tatiana E. Erova,[a] Jason A. Rosenzweig,[b] Jian Sha,[a,e] Giovanni Suarez,[a] Johanna C. Sierra,[a] Michelle L. Kirtley,[a] Christina J. van Lier,[a] Maxim V. Telepnev,[c] Vladimir L. Motin,[a,c,d,e,f] Ashok K. Chopra[a,d,e,f]

Departments of Microbiology and Immunology[a] and Pathology,[c] Sealy Center for Vaccine Development,[d] Institute of Human Infections and Immunity,[e] and Galveston National Laboratory,[f] University of Texas Medical Branch, Galveston, Texas, USA; Department of Biology, Center for Bionanotechnology and Environmental Research, Texas Southern University, Houston, Texas, USA[b]

Plague caused by *Yersinia pestis* manifests itself in bubonic, septicemic, and pneumonic forms. Although the U.S. Food and Drug Administration recently approved levofloxacin, there is no approved human vaccine against plague. The capsular antigen F1 and the low-calcium-response V antigen (LcrV) of *Y. pestis* represent excellent vaccine candidates; however, the inability of the immune responses to F1 and LcrV to provide protection against *Y. pestis* F1$^-$ strains or those which harbor variants of LcrV is a significant concern. Here, we show that the passive transfer of hyperimmune sera from rats infected with the plague bacterium and rescued by levofloxacin protected naive animals against pneumonic plague. Furthermore, 10 to 12 protein bands from wild-type (WT) *Y. pestis* CO92 reacted with the aforementioned hyperimmune sera upon Western blot analysis. Based on mass spectrometric analysis, four of these proteins were identified as attachment invasion locus (Ail/OmpX), plasminogen-activating protease (Pla), outer membrane protein A (OmpA), and F1. The genes encoding these proteins were cloned, and the recombinant proteins purified from *Escherichia coli* for immunization purposes before challenging mice and rats with either the F1$^-$ mutant or WT CO92 in bubonic and pneumonic plague models. Although antibodies to Ail and OmpA protected mice against bubonic plague when challenged with the F1$^-$ CO92 strain, Pla antibodies were protective against pneumonic plague. In the rat model, antibodies to Ail provided protection only against pneumonic plague after WT CO92 challenge. Together, the addition of *Y. pestis* outer membrane proteins to a new-generation recombinant vaccine could provide protection against a wide variety of *Y. pestis* strains.

Yersinia pestis, the causative agent of bubonic, septicemic, and pneumonic plague, is a Gram-negative zoonotic pathogen, and human infections are often acquired through the infected-flea vector carried by domestic and/or wild animals (1). The microorganism possesses multiple virulence determinants that are both chromosomally and extrachromosomally encoded on its three plasmids: pCD1, pMT1/pFra, and pPCP1/pPla (2). Although bubonic plague, inflicted by flea bites, in humans represents the most common form worldwide (http://www.gov.mb.ca/health /publichealth/cdc/protocol/plague.pdf), it can lead to secondary septicemic and pneumonic plague, with a nearly 100% mortality rate. Primary pneumonic plague results by inhaling aerosolized droplets charged with *Y. pestis*, and the organism can then rapidly spread from person to person, possibly leading to mass causalities (http://www.cdc.gov/ncidod/dvbid/plague/info.htm).

Presently, there is no licensed plague vaccine in the United States (3), which is alarming in the light of epidemics and potential bioterrorism threats associated with this lethal pathogen. The current subunit vaccines under investigation include the capsular antigen F1 and the low-calcium-response V antigen (LcrV). LcrV is part of the type 3 secretion system (T3SS) and is encoded by the pCD1 plasmid (3). F1 and LcrV vaccines are tested as either purified individual antigens or as a fusion of these proteins (F1-V or V-F1) (4–6; see also reference 3 and references therein). Although efficacious in rodents and some nonhuman primate models (4–6; see also reference 3 and references therein), these recombinant vaccines would not be as efficacious against F1$^-$ strains of *Y. pestis* that exist in nature and are as virulent as the wild-type (WT) *Y.*

pestis (7, 8). However, since LcrV is highly immunogenic and represents a protective antigen of *Y. pestis*, anti-LcrV antibodies alone would provide significant protection to the host against challenge with the F1$^-$ strains even if antibodies to F1 were inconsequential. Indeed, antibodies to recombinant LcrV alone were shown to provide protection in mice against both bubonic and pneumonic plague caused by encapsulated and F1$^-$ *Y. pestis* strains (9), whereas antibodies to F1 protected animals from the F1$^+$ strain (10). Likewise, it was shown that an F1-V fusion protein, as well as F1 or LcrV immunogens, individually protected mice against pneumonic plague induced by WT or its F1$^-$ virulent isogenic *Y. pestis* strain (11). In the same vein, Heath et al. (12) reported the protection of mice against bubonic and pneumonic plague by antibodies to an F1-V fusion protein against challenge with both F1$^+$ and F1$^-$ strains of *Y. pestis*. However, they noted that immunization of animals with the F1-V fusion protein led to better protection compared to mice that were vaccinated with either F1 or LcrV antigen alone against the F1$^+$ strain (12).

More importantly, the sequence of LcrV has diverged in various *Y. pestis* strains, and thus antibody responses to one variant may not provide optimal protection against *Y. pestis* strains that have LcrVs with varied amino acid sequences (7, 8). In one study, an LcrV variant (LcrV5214), mutated for 5 amino acid residues (thus unable to interact with the Toll-like receptor 2), was expressed in a live-attenuated *Salmonella enterica* Typhimurium strain; when this recombinant *Salmonella* strain was tested as a potential oral vaccine in mice, it did not protect animals against developing bubonic plague (13). Later, Sun et al. (14) also indicated that when the native LcrV-encoding gene on the pCD1 plasmid was replaced with its variant LcrV2345 (with similar 5-amino-acid substitutions, as described above) in *Y. pestis* KIM6 strain (deleted of the pigmentation [*pgm*] locus) and both were used to induce bubonic and pneumonic plague in mice, no difference in bacterial virulence was observed between the two strains. Thus, immune responses only to F1 and LcrV to provide protection against *Y. pestis* F1⁻ strains or against strains that harbor variants of LcrV are a real concern.

Considering the limitations associated with LcrV and F1 antigens mentioned above, it seems necessary that additional antigens be incorporated into novel recombinant plague vaccine preparations. In that vein, we used immunoblotting with rat hyperimmune sera, obtained from WT *Y. pestis* CO92-infected and levofloxacin-rescued animals, to identify novel plague antigens that could be incorporated into a new-generation subunit plague vaccine. Using mass spectrometric analysis, we identified four outer membrane protein (OMP) antigens, namely, F1, attachment-invasion locus (Ail/OmpX), plasminogen-activating protease (Pla), and outer membrane protein A (OmpA), to which the rat antisera reacted. Since anti-F1 antibodies are protective against pneumonic plague in a mouse model (see reference 3 and references therein), we primarily focused our studies on the other three OMPs (Ail/OmpX, OmpA, and Pla), of which two (Ail/OmpX and Pla) are bona fide virulence factors of *Y. pestis* (15, 16).

It is important to mention here that the genetic background of the mouse strains does contribute to their susceptibility or resistance to developing plague. For example, both F1 and fimbrial protein PsaA were needed for the full virulence of *Y. pestis* in terms of inducing bubonic and pneumonic plague in C57BL/6J mice (17). However, the Δ*caf1* mutant exhibited a more attenuated phenotype in developing bubonic plague in C57BL/6J mice compared to BALB/cJ animals (17). Because of the diversity in the genetic makeup of humans, we evaluated whether the active immunization of outbred Swiss-Webster mice and Brown Norway rats with purified, recombinant Ail/OmpX, OmpA, and Pla could generate protective antibodies against bubonic and pneumonic plague induced by both F1⁻ and WT CO92 strains.

Pla is a 312-amino-acid aspartate multifunctional protease encoded by the pPCP1 plasmid and has been shown to enhance bacterial adherence to the extracellular matrix (18, 19). In addition, Pla activates circulating human plasminogen into plasmin that degrades components of the extracellular matrix (20, 21), thus facilitating bacterial dissemination to peripheral organs. Pla also modulates bacterial virulence by degrading *Yersinia* OMPs and their secretion (22, 23) and by interacting with lipopolysaccharide, a major component of the outer membrane in Gram-negative bacteria (24).

Attachment invasion locus (Ail/OmpX) protein belongs to the Ail/Lom family of OMPs and provides protection to *Y. pestis* from complement-dependent killing (25). Furthermore, Ail is a dominant adhesion molecule that plays an important role in the delivery of T3SS effectors into targeted host cells during infection (26). In both rat and mouse bubonic plague models of infection, a Δ*ail* mutant exhibited reduced systemic spread as well as greatly attenuated virulence (greater in rats than in mice); however, a productive adaptive immune response was generated after a high-dose challenge, as evidenced by significant serum antibody levels against the *Y. pestis* F1 antigen (16). Likewise, Kolodziejek et al. reported that the deletion of the *ail* gene from *Y. pestis* CO92 led to a highly attenuated mutant strain in a rat model of pneumonic plague (27). In a *Y. pestis* KIM5 background strain (deleted of the *pgm* locus), Ail was shown to be more important for host cell binding, in inducing host cell cytotoxicity, and for virulence in a mouse model of infection when the bacteria were given by the intravenous route than Pla and that Pla mediation of Yop secretion was independent of its protease activity (28). Ail specifically binds fibronectin, an extracellular matrix protein, and possibly acts as a bridge receptor between Ail and the host cells (29). Interestingly, anti-fibronectin antibodies inhibited the KIM5-mediated cytotoxicity of the host cells in an Ail-dependent fashion, demonstrating the necessity of this interaction for Yop delivery into the host (29).

OmpA plays a significant role in anchoring the outer membrane to the cell wall peptidoglycan and has pore-forming activity, producing nonspecific diffusion channels (30, 31). Indeed, OmpA in several pathogens has been shown to mediate resistance to complement and antibacterial peptides, as well as play a role in invasion and intracellular survival (32–34). Recently, OmpA was identified as a prosurvival factor for macrophage-engulfed *Y. pestis*, and a Δ*ompA* mutant strain was outcompeted by the WT strain in a mouse coinfection assay, suggesting a role it may play during dissemination within the host (35).

The present study was undertaken to specifically demonstrate whether antibodies to Ail, Pla, and OmpA provide protection to animals against bubonic and pneumonic plague caused by WT *Y. pestis* CO92 and its F1⁻ mutant strain.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in the present study are listed in Table 1. *Y. pestis* strains were grown at 28°C in heart infusion broth (HIB; Bacto, Sparks, MD) or on sheep blood agar plates in the Centers for Disease Control and Prevention-approved select agent laboratory. *Escherichia coli* cultures were grown at 37°C in Luria-Bertani (LB) broth and on LB agar plates. The antibiotic kanamycin was used at a working concentration 50 μg/ml.

Reagents and commercial antigens. Restriction enzymes, *Taq* DNA polymerase, and T4 DNA ligase were purchased from New England BioLabs (Beverly, MA). A His Spin^Trap kit and columns charged with Ni²⁺-Sepharose were purchased from GE Healthcare Biosciences Corp. (Piscataway, NJ). Imject Alum used as an adjuvant was purchased from Thermo Scientific Pierce Biotechnology (Rockford, IL). The F1-LcrV (F1-V) fusion protein was obtained from the Biodefense and Emerging Infections Research Resources Repository, Manassas, VA.

DNA isolation and PCR assays. *Y. pestis* CO92 genomic DNA (gDNA) was isolated by utilizing a DNeasy tissue kit from Qiagen (Valencia, CA). PCR assays were performed under standard conditions as follows: an initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 65°C, and extension for 30 s at 72°C, with a final extension for 7 min at 72°C. Amplified products were purified using a PCR purification kit (Qiagen). Plasmid DNA was isolated using a QIAprep spin miniprep

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Y. pestis		
CO92 BEI	Fully virulent WT strain	BEI[b]
Δ*caf1*	Deletion mutant of the *caf1* gene (F1)	39
E. coli BL21(DE3)	F⁻ *ompT gal dcm lon hsdS*$_B$(r$_B^-$ m$_B^-$) λ(DE3 [*lacI lacUV5*-T7 gene 1 *ind1 sam7 nin5*])	Novagen
Plasmids		
pET-30a(+)	T7 promoter-based prokaryotic expression vector; Km^r	Novagen
pET-20b(+)	T7 promoter-based prokaryotic expression vector; Ap^r	Novagen
pET-30/*ail*	*ail* gene of *Y. pestis* cloned at BglII/SalI sites of pET-30a(+) vector for purification of Ail protein; Km^r	This study
pET-30/*ompA*	*ompA* gene of *Y. pestis* cloned at NdeI/XhoI sites of pET-30a(+) vector for purification of OmpA protein; Km^r	This study
pET-20/*caf1*	*caf1* gene of *Y. pestis* cloned at NdeI/XhoI sites of pET-20b(+) vector for purification of F1 protein; Ap^r	This study
pET-20/*pla*	*pla* gene of *Y. pestis* cloned at NdeI/XhoI sites of pET-20b(+) vector for purification of Pla protein; Ap^r	38

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance.
[b] BEI, The Biodefense and Emerging Infections Research Resources Repository, Manassas, VA.

kit (Qiagen). All primers (Table 2) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Transformation of *E. coli* BL21(DE3) strain. Preparation of competent cells and plasmid DNA transformations of *E. coli* BL21(DE3) strain were performed by using the Z-Competent transformation kit from Zymo Research (Orange, CA), as described previously (36).

Hyperimmune sera from rats infected with WT *Y. pestis* CO92 and rescued with levofloxacin. Brown Norway rats (*n* = 9/group) were challenged with WT CO92 (10 50% lethal doses [LD$_{50}$] of 5,000 CFU administered intranasally [i.n.]) and then given levofloxacin (5 mg/kg/day given intraperitoneally [i.p.]), the minimal protective dose, at 24, 36, 42, and 48 h postinfection (p.i.) for 6 days. At 34 days p.i., the surviving animals were rechallenged with 10 LD$_{50}$ of WT CO92. The animals were bled after 64 days, and the immune sera from the survivors were used for performing Western blot analysis against total cell lysates of WT CO92. Sera from uninfected but levofloxacin-treated animals served as a negative control.

Western blot analysis. Whole-cell lysates of WT CO92 were subjected to sodium dodecyl sulfate (SDS)–10 to 16% gradient polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting with rat hyperim-

mune or preimmune sera (see above) at a dilution of 1:1,000. After treatment of the membranes with goat anti-rat secondary antibodies conjugated with horseradish peroxidase (HRP), enhanced chemiluminescence (ECL) substrate was used to visualize the protein bands by using an ECL Western blotting detection reagent kit (GE Healthcare).

Passive transfer of hyperimmune rat sera to naive animals. The hyperimmune rat sera obtained from *Y. pestis*-infected and levofloxacin-rescued rats that were given the antibiotic regimen 24 h p.i. for 6 days (see above) were pooled and used for the present study. Naive rats were administered 100 μl of the hyperimmune sera by the i.p. route. After 4 h, the passively immunized rats were i.n. challenged with 20 LD$_{50}$ of WT *Y. pestis* CO92, and the mortality in animals was monitored. As controls, naive rats received either preimmune rat sera or phosphate-buffered saline (PBS).

Mouse passive antibody transfer. Swiss-Webster mice (*n* = 15) were i.n. infected with 1 LD$_{50}$ of WT *Y. pestis* CO92 (500 CFU). After 30 days, we terminally bled the survivors (*n* = 8), and their sera were used for the passive immunization of naive mice. Briefly, 100 μl of pooled immune or preimmune sera was injected i.p. into naive mice (*n* = 10/group). After 4 h, passively immunized mice were i.n. challenged with 10 LD$_{50}$ of WT CO92 (5,000 CFU) and observed for survival.

Differences in the immune responses of mice and rats. WT *Y. pestis* CO92 was i.n. administered to animals (mice, *n* = 10/group; rats, *n* = 9/group) at the target challenge dose of 10,000 CFU, which correlated with 20 LD$_{50}$ for both the rodent species. At 24 h p.i., levofloxacin was administered once daily (10 mg/kg) for 6 days via the i.p. route. The control animal groups did not receive the antibiotic but were infected. All of the animal studies were performed with either Swiss-Webster mice or Brown Norway rats.

Animal survival was monitored and recorded for 29 days p.i. On day 30 of the study, all survivors were rechallenged with WT CO92 via the i.n. route (20 LD$_{50}$) and monitored for an additional 34 days. At the same time point, animals in the rechallenge control groups were i.n. infected for the first time with 20 LD$_{50}$ of WT CO92.

Cloning of *ail*, *ompA*, *pla*, and *caf1* genes in *E. coli* and purification of the recombinant proteins. Using the pET-30a(+) vector, plasmid constructs containing the PCR-amplified *ail* and *ompA* genes (using the primers listed in Table 2) were generated. The amino-terminal (NH$_2$) histidine (His)-tagged Ail and OmpA antigens from *E. coli* BL21(DE3) were purified by using the His Spin^{Trap} kit (GE Healthcare) according to the recommended protocol under denaturing conditions with 8 and 4 M urea, respectively. Briefly, recombinant *E. coli* cultures were grown to log phase in LB broth at 37°C with agitation. Cells were then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. After sonication and centrifugation, membrane proteins were solubilized in the binding buffer containing 4 to 8 M urea and loaded onto the His column. After a thorough washing of the column (3×), the bound proteins were eluted with 500 mM imidazole and urea. The purity of recombinant proteins was examined by Coomassie blue and SYPRO-Ruby staining (Bio-Rad, Hercules, CA), and the concentration of antigens was determined by spectrophotometric readings of the optical density at 280 nm (OD$_{280}$) and a BCA protein assay kit (Thermo Scientific), which is based on Lowry's method (37).

TABLE 2 Primers used in this study

Primer[b]	Sequence (5′–3′)[a]	Purpose
ail/BglII^F	TCAT<u>AGATCT</u>AATGAATAAGACATTACTGGTCTCT	Cloning of the *ail* gene into the pET-30a(+) vector
ail/SalI^R	GGTC<u>GTCGAC</u>TTAGAACCGGTAACCCGCGCCAAGC	Cloning of the *ail* gene into the pET-30a(+) vector
ompA/NdeI^F	AGC<u>CATATG</u>AAAAAGACAGCTATCGCATTAGC	Cloning of the *ompA* gene into the pET-30a(+) vector
ompA/XhoI^R	AAC<u>CTCGAG</u>AGCCTGTGGCTGAGTCACAAC	Cloning of the *ompA* gene into the pET-30a(+) vector
caf1/NdeI^F	CAC<u>CATATG</u>AAAAAAATCAGTTCCGTTATCG	Cloning of the *caf1* gene into the pET-20b(+) vector
caf1/XhoI^R	CAC<u>CTCGAG</u>TTGGTTAGATACGGTTACGGTTACAG	Cloning of the *caf1* gene into the pET-20b(+) vector

[a] Underlining indicates restriction endonuclease site.
[b] F, forward; R, reverse.

Likewise, the NH_2-terminal His-tagged Pla was purified in a single-step affinity chromatography using Ni^{2+}-Sepharose chromatography under denaturing conditions, as described previously (38). This antigen was insoluble and did not activate plasminogen. On the contrary, the carboxyl-terminal His-tagged F1 was Ni^{2+}-Sepharose affinity chromatography purified in a soluble form under native conditions. The purity of these antigens was also examined by Coomassie blue and SYPRO-Ruby staining of SDS–12% polyacrylamide gels.

Continuous-elution electrophoresis and mass spectrometry. A saturated 10-ml culture of *Y. pestis* CO92 was grown in HIB medium at 28°C with agitation (180 rpm) from a frozen −80°C stock. Subcultures in 90 ml of fresh HIB medium were incubated for 2 h under the same conditions. The temperature of the culture was then shifted to 37°C for the next 4 h of incubation. The bacterial cells were pelleted and then resuspended in 10 ml of SDS-PAGE sample buffer containing β_2-mercaptoethanol. The sample was heated at 100°C for 10 min in a dry bath and plated to confirm the sterility of the sample. An aliquot of the sample (1 ml) was loaded onto a 10% polyacrylamide gel and electrophoresed under conditions specified by the manufacturer by using a Model 491 Prep Cell (Bio-Rad), which is designed to purify proteins from complex mixtures by continuous-elution electrophoresis. Once the blue dye of the sample migrated through 3/4 of the gel, the proteins were eluted from the column using elution buffer (50 mM ammonium formate, 0.01% SDS [pH 8.0]), and 4 ml of each fraction (for a total of 150 to 200 ml) was collected. The samples were concentrated to 1/4 of the volume in a Savant SpeedVac (Biodirect, Inc., Taunton, MA) and then dialyzed against PBS. An aliquot of the samples was subjected to SDS-PAGE and SYPRO-Ruby staining. In a parallel gel, Western blot analysis was performed with hyperimmune rat sera to identify immunoreactive bands. The matching protein bands were manually removed from the stained gel, trypsin digested, and subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) at the Protein Chemistry Core Laboratory at the University of Texas Medical Branch (UTMB).

Immunization of animals. Five- to six-week-old female Swiss-Webster mice ($n = 10$/group; Taconic Farms, Germantown, NY) and 5-week-old female Brown Norway rats ($n = 6$/group; Harlan Laboratories, Houston, TX) were immunized on day 0, followed by two boosters (given 2 weeks apart) with the purified antigens at concentrations of 20 µg per animal after being mixed with the Imject Alum (1:1) by the intramuscular (i.m.) route. The control animals received adjuvant only. Blood was drawn prior to immunization and 1 week after the second booster. All of the animal immunization protocols were performed in an Animal Biosafety Level 2 (ABSL-2) facility within the Galveston National Laboratory (GNL), UTMB.

Animal challenges. One week after the second antigen booster, the immunized animals were moved to an ABSL-3 facility within the GNL and infected by the i.n. and s.c. routes with WT *Y. pestis* CO92 or an isogenic Δ*caf1* mutant of *Y. pestis* CO92 (39). All of the animal challenge experiments were performed in the ABSL-3 facility. Briefly, mice and rats were anesthetized with a mixture of xylazine-ketamine or isoflurane. The animals were observed daily for signs of morbidity and mortality during the course of the experiment. For mice immunized with Ail, OmpA, and Pla, we challenged them with 500 LD_{50} (2.5×10^4 CFU) of the Δ*caf1* mutant of *Y. pestis* CO92 by the s.c. route to induce bubonic plague (39). Likewise, we infected immunized mice with 15 LD_{50} (i.n., 7,500 CFU) of the Δ*caf1* mutant to induce pneumonic plague.

For rats immunized with F1-V antigen, we used an i.n. challenge dose of 5×10^6 CFU for infection, representing 10,000 LD_{50}, of WT CO92. For rats immunized with Ail, OmpA, and Pla antigens, we used an i.n. challenge dose of 4×10^3 to 5×10^3 CFU for infection, which represented 8 to 10 LD_{50}, and an s.c. dose of 7×10^2 to 8×10^2 CFU, which corresponded to 7 to 8 LD_{50} of WT CO92. All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at UTMB.

Analysis of antibody production by ELISA. For enzyme-linked immunosorbent assays (ELISAs), 96-well microtiter plates (Evergreen Scientific, Los Angeles, CA) were coated with 10 ng of Ail, OmpA, Pla, F1, or F1-V antigen/well at 4°C overnight. After blocking and washing (40), sera from naive and immunized mice and rats were serially diluted and incubated with the affixed antigens in the microtiter plates for 1 h at room temperature. After several washes, HRP-conjugated goat anti-mouse/rat secondary antibodies were added to the wells at a dilution of 1:10,000 for 1 h at room temperature, followed by several washes and the addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB). After a 20-min colorimetric development period, the reaction was quenched by the addition of 2 N H_2SO_4, and the plate/well absorbance was read at OD_{450} using a spectrophotometer.

For Western blot analysis, purified antigens (OmpA, Ail, Pla, and F1-V; 100 ng) were subjected to SDS–12% PAGE and immunoblotting. The blots were probed with sera to specific antigens.

Antibodies to whole bacteria in the hyperimmune rat sera. *Y. pestis* CO92 cells were grown to log phase and, after centrifugation, were resuspended in PBS at a concentration of 5×10^9 CFU/ml. Subsequently, the low-binding 96-well microtiter plates were coated with poly-L-lysine (10 µg/ml in PBS, 50 µl/well) for 30 min at room temperature, followed by the addition of 50 µl of the bacterial suspension/well, after washing off the residual lysine. The plates were centrifuged at 1,000 rpm for 5 min at 4°C, followed by the addition of 50 µl of a 0.5% glutaraldehyde solution/well and incubation for 15 min. The glutaraldehyde solution was gently removed, and the wells were washed several times with PBS in the biological safety hood in the select agent laboratory. The wells were then treated with 200 µl/well of a solution of 0.1 M glycine–0.1% bovine serum albumin (BSA) in water, followed by incubation for 30 min at room temperature. The wells were washed with PBS, followed by blocking of the wells of the plates with 200 µl of PBS–1% BSA/well for 1 h at room temperature. After the blocking solution was removed, 100 µl of the primary antibodies (naive versus hyperimmune sera, 10-fold diluted) in PBS–0.1% BSA were added for 1 h. After five washes with PBS–0.05% Tween 20 solution, 100 µl of HRP-conjugated secondary antibodies (diluted 1:10,000 in PBS–0.1% BSA) was added for 1 h. After thorough washing, 100 µl of the substrate (TMB) was added as described above. The reaction was quenched by adding 50 µl of 2 N H_2SO_4, and the color intensity was read at OD_{450}.

Statistics. Wherever appropriate, data were analyzed using unpaired Student *t* test. The animal data were analyzed by using either the Fisher exact test, Kaplan-Meier's survival estimates, or an unpaired Student *t* test with *P* values of ≤0.05 considered significant.

RESULTS

Brown Norway rats infected with WT *Y. pestis* CO92 and rescued by levofloxacin were protected against developing subsequent pneumonic plague. With the goal of producing hyperimmune sera raised against plague outer membrane antigens, we challenged rats with WT CO92 (10 LD_{50} by the i.n. route) and then administered levofloxacin (5 mg/kg/day, the MIC) at 24, 36, 42, and 48 h p.i. for 6 days. As noted (Fig. 1A), 100% of the animals survived initial challenge when the levofloxacin treatment was delayed up to 42 h. On the contrary, >50% of the rats died when the antibiotic treatment was delayed to 48 h after infection. All of the levofloxacin-treated groups of rats (24 to 42 h, $P = 0.0011$; 48 h, $P = 0.0032$) were significantly protected based on the Kaplan-Meier's survival estimates compared to the control group of animals that were infected but not treated with levofloxacin. As expected, all of the control rats died by day 6 after initial infection (Fig. 1A).

Upon rechallenge of the survivors on day 34, 90 to 100% of the animals were still protected in those groups that received levofloxacin 24 to 42 h p.i. ($P < 0.0055$ to 0.0001), whereas this pro-
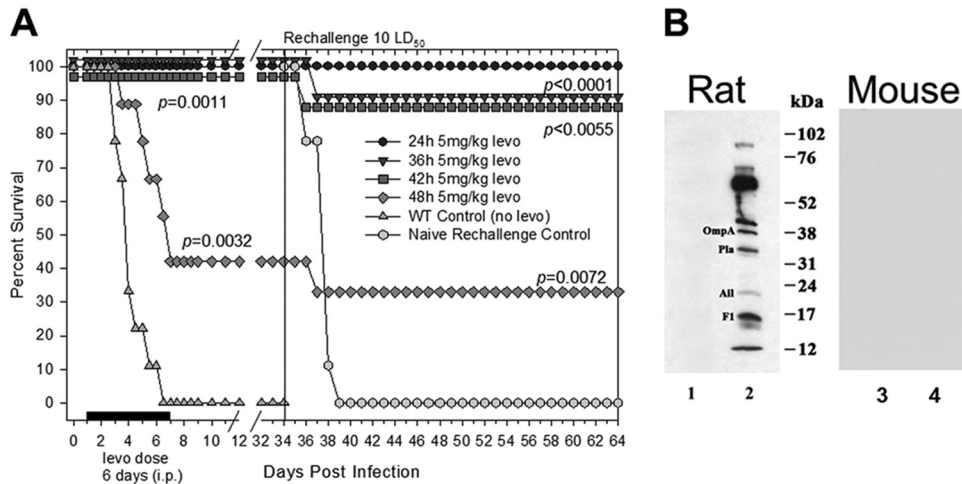
FIG 1 (A) Delayed levofloxacin (levo) treatment of rats infected intranasally with 10 LD$_{50}$ of WT *Y. pestis* CO92 was protective against developing pneumonic plague. Antibiotic treatment (5 mg/kg/day) was initiated by 24, 36, 42, and 48 h p.i. and continued for 6 days thereafter. The solid black bar on the *x* axis represents 6 days of antibiotic treatment starting 24 h p.i. Animals were rechallenged with 10 LD$_{50}$ of WT CO92 on day 34 (indicated by a vertical line) and monitored for another 30 days. The data were analyzed statistically using Kaplan-Meier survival estimates, and the *P* values are indicated. (B) Detection by immunoblot analysis and identification of OmpA, Pla, Ail, and F1 target antigens by MALDI-TOF (lane 1, preimmune; lane 2, immune) is shown from sera of rats infected by the i.n. route with 10 LD$_{50}$ of WT *Y. pestis* CO92 and rescued by levofloxacin (designated as rat). No protein bands were detected when pooled sera from *Y. pestis*-infected and levofloxacin-rescued mice were used (designated as mouse) under conditions identical to those for rat sera. Lane 3, preimmune mouse sera; lane 4, immune mouse sera.

tection dropped to 35% in the group of rats in which the levofloxacin treatment was delayed to 48 h. However, this protection was still statistically significant compared to the control animals ($P = 0.0072$). It should be noted that no levofloxacin was given to the animals after rechallenge. The age/sex-matched control infected rats died of plague by day 5 (Fig. 1A). These data demonstrated that levofloxacin-treated animals developed a protective immunity to *Y. pestis*. Similar results were observed when animals were administered levofloxacin at 10 mg/kg/day at the same time points (data not shown).

To then determine whether the hyperimmune sera from these rats would react with WT CO92 proteins, we performed immunoblot analysis. We used pooled sera from rats that were infected with *Y. pestis* CO92 and given levofloxacin 24 h p.i. (Fig. 1A). In doing so, we detected 10 to 12 discrete bands in the size range of 12 to 102 kDa that reacted with rat immune (Fig. 1B, lane 2) but not preimmune sera (lane 1). Clearly, genes encoding these target antigens were of interest to us.

Passive transfer of hyperimmune rat sera to naive rats was protective against inducing pneumonic plague. To demonstrate whether passively transferred hyperimmune rat sera, used for Western blot analysis, also provided protection to naive rats against developing pneumonic plague, animals were administered either immune or preimmune sera via the i.p. route prior to i.n. challenge with the WT *Y. pestis* CO92. Prior to performing this passive immunization experiment, we determined the antibody titer in the hyperimmune rat sera against the whole *Y. pestis* cells by performing whole-cell ELISA (Fig. 2A). In these passive immunization studies, the animals were never treated with levofloxacin. As shown in Fig. 2A, significant antibody titers (1:10^8) were observed in *Y. pestis*-infected and levofloxacin-rescued rats compared to the preimmune control sera.

At 4 h after administration of the hyperimmune or preimmune sera, rats were i.n. challenged with 20 LD$_{50}$ of WT CO92, and all

animals that either did not receive any sera or were injected with the preimmune sera died by day 4 (Fig. 2B). However, rats that received hyperimmune sera demonstrated 55% protection until day 19, which then dropped to 44%, still a significant difference relative to the above-mentioned two control groups ($P < 0.0001$). On day 23, we rechallenged the surviving animals that received hyperimmune sera with the same LD$_{50}$ of CO92, and no further deaths occurred (Fig. 2B). The rationale of this study was 2-fold and included (i) determination of whether passively administered *Y. pestis*-specific hyperimmune rat sera could protect naive animals against pneumonic plague and (ii) determination of whether surviving animals could mount an independently active immune response against the plague bacterium that could protect rats during a rechallenge. Animals from the same batch and age that received preimmune sera served as a negative control for the rechallenge study, and all of them died (as expected) (Fig. 2B). These data demonstrated that immunoreactive antigens detected by Western blotting (Fig. 1B) were capable of generating protective antibodies which prevented rats from developing pneumonic plague. Interestingly, parallel mouse studies indicated that passive transfer of sera from *Y. pestis*-infected mice (given 1 LD$_{50}$ by the i.n. route [see Materials and Methods]) to naive mice did not provide any protection (data not shown).

Differences in the adaptive immune response of mice and rats during pneumonic plague. Mice and rats were i.n. challenged with 20 LD$_{50}$ of WT CO92 in parallel. After 24 h p.i., animals received levofloxacin at 5 to 10 mg/kg/day via the i.p. route for 6 days. As expected, both the mouse and the rat untreated challenge control animals (without levofloxacin) died by day 4 p.i. In sharp contrast, 100% of the mice and rats survived when given levofloxacin 24 h p.i., confirming that this concentration of the antibiotic was indeed protective for both animal models (Fig. 3). This level of survival was statistically significant compared to the chal-
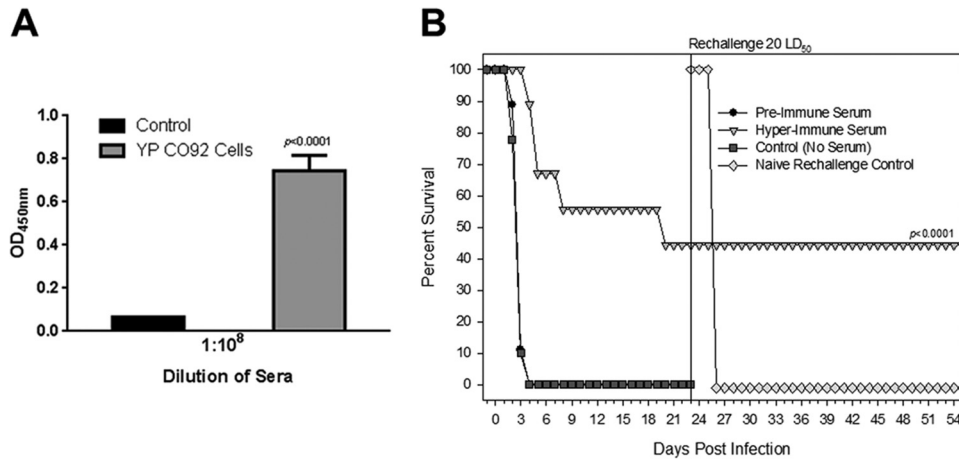
FIG 2 (A) Evaluation of antibody titers against the whole WT CO92 bacterial cells by ELISA. The hyperimmune rat sera obtained after *Y. pestis* infection and rescuing of the animals with levofloxacin given 24 h p.i. were used for ELISA. The sera were pooled from all of the surviving animals, and the experiment was performed in triplicate. The arithmetic means $\pm$ the standard deviations are plotted. The pooled preimmune sera were used as a control. The sera were 10-fold diluted to determine endpoint antibody titers. (B) Passive transfer of pooled hyperimmune rat sera to naive rats provided protection to the latter when challenged with 20 LD_{50} of WT *Y. pestis* CO92. On day 23, the surviving rats were rechallenged with the same LD_{50} of WT CO92 (indicated by a vertical line). The data were statistically analyzed by using Kaplan-Meier survival estimates, and the P value is provided. Animals that were injected with preimmune sera served as a negative control. For rechallenge, we used same age- and sex-matched naive rats as a control to demonstrate that the given dose of *Y. pestis* led to 100% mortality due to pneumonic plague.

lenge control group beginning on day 3, as determined by the Kaplan-Meier and Fisher exact tests ($P < 0.0001$).

On day 30 p.i., survivors (both mice and rats) were rechallenged to demonstrate the development of protective immunity against reinfection with 20 LD_{50} of WT CO92. The age-matched naive control animals were similarly infected, and all succumbed to plague as expected. Importantly, levofloxacin-treated rats demonstrated 100% survival after rechallenge ($P < 0.0001$ relative to

FIG 3 Mice and rats that were infected with 20 LD_{50} of WT CO92 and then treated with levofloxacin at 10 mg/kg/day starting at 24 h p.i. for 6 consecutive days were 100% protected from developing pneumonic plague. The solid black bar along the *x* axis represents levofloxacin treatment of animals 24 h after infection, and the antibiotic regimen was given over a period of 6 days. On day 30 (indicated by a vertical line), the survivors were rechallenged with the same LD_{50} of WT CO92. Age- and sex-matched animals were used as rechallenge controls. The data were statistically analyzed by using Kaplan-Meier survival estimates, and the P values are reported.

the control group), whereas only 10% of the levofloxacin-treated mice survived rechallenge (Fig. 3).

Overall, these data tend to suggest key differences in the immune responses of mice versus rats to pneumonic plague. We speculate that mice do not develop as robust an adaptive immune response as do rats for protection against subsequent plague infections. Investigations of differences between infected B- and T-cell responses of the two species have not yet been published, representing a gap in our understanding of the rodent models of pneumonic plague. Our speculation was based on the fact that sera from *Y. pestis*-infected and levofloxacin-rescued mice (Fig. 3) did not react with any WT CO92 protein bands upon Western blot analysis at a dilution of 1:1,000 (Fig. 1B). This was in sharp contrast to the hyperimmune sera obtained on day 34 and 64 (Fig. 1A) from rats which reacted with 10 to 12 discrete bands. Consequently, we used both rodent species in our studies.

Identification of immunoreactive antigens of WT CO92 that reacted with rat hyperimmune sera. Based on continuous-elution electrophoresis, Western blot analysis of isolated proteins, and mass spectrometry, we identified four *Y. pestis* CO92 proteins (<40 kDa) that reacted strongly with our rat hyperimmune sera (Fig. 1B). One of them was F1 (16 kDa), and the remaining three were Ail/OmpX (20 kDa), Pla (34 kDa), and *Salmonella*-like OmpA (38 kDa) (Fig. 4). It was of interest to note that F1 migrated more slowly than expected, representing the aggregative nature of this protein (41). The bands which migrated close to 17 or 14 kDa on the Western blot (Fig. 4) also represented Ail/OmpX, based on MALDI-TOF analysis. Finally, the bands which migrated between 36 and 52 kDa were identified as DNA-directed RNA polymerase alpha subunit (36 kDa), OmpC (41 kDa), and elongation factor Tu ($\sim$50 kDa).

Plasmid constructions and purification of *Y. pestis* antigens. The genes for *ail*, *ompA*, *pla*, and *caf1* were cloned into pET expression vectors with appropriate restriction sites and sequenced (Tables 1 and 2). Ail, OmpA, and Pla consist of 179, 363, and 312
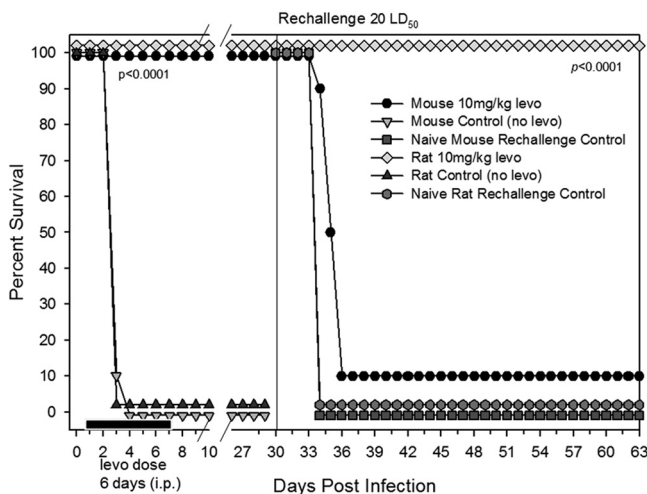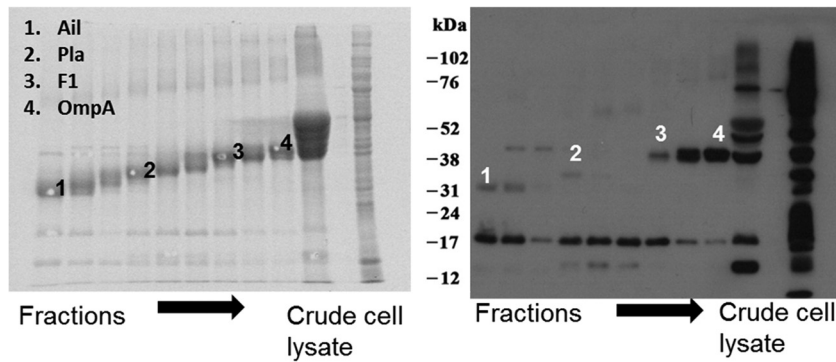
FIG 4 Continuous elution SDS–10% PAGE using a Model 491 Prep Cell. Whole-cell lysates of WT CO92 were run on the gel, and the fractions were collected. Every four fractions were pooled, concentrated, dialyzed, subjected to SDS–12% PAGE, and stained with SYPRO-Ruby (left panel). An aliquot of the starting crude material was also subjected to electrophoresis. A parallel gel was subjected to Western blot analysis using hyperimmune rat sera (Fig. 1B) (right panel). The Western blot bands that corresponded to the SYPRO-Ruby-stained protein bands were picked and subjected to MALDI-TOF for identification. The identity of spots is indicated. The presence of multiple bands on the Western blot represented either aggregated or degraded version of the same proteins, as confirmed by mass spectrometry of several bands. The migration of molecular mass markers is also shown.

amino acid residues, respectively, and were purified by affinity chromatography under denaturing conditions with urea because of their insoluble nature. F1 consists of 178 amino acid residues and was purified under native conditions.

Protective effects of antibodies to F1, LcrV, Pla, OmpA, and Ail/OmpX against *Y. pestis* infection in a mouse model. Before evaluating protection conferred by Pla, OmpA, or Ail/OmpX recombinant proteins, we first tested the efficacy of F1 and LcrV subunit vaccines in providing protection to mice against pneumonic plague to establish a comparative benchmark. Mice were immunized via the i.m. route (20 μg of purified F1-V or F1 in alum) on days 0, 15 and 30 and then challenged after 45 days. We observed 50% protection in mice immunized with F1 after i.n. challenge with 100 LD_{50} of WT *Y. pestis* CO92, which corresponded to high F1 antibody titers in the animal sera (Fig. 5), whereas this protection was 100% when the animals were immunized with F1-V.

Following i.m. immunizations with OmpA, Ail/OmpX, and Pla, mice mounted antibody responses against all three antigens,

although the titers varied. The titers of anti-Pla and anti-OmpA antibodies were the lowest, while the anti-Ail/OmpX antibody titers developed were similar to that of F1-V antigen (Fig. 6A). These antibodies specifically reacted with their respective purified antigens on Western blots (see insets). Mice immunized with the aforementioned antigens were subsequently infected via the s.c. route with 500 LD_{50} (which corresponds to approximately 75 to 100 LD_{50} of the WT *Y. pestis* CO92) of our Δ*caf1* mutant strain of *Y. pestis* CO92 (39). The Δ*caf1* mutant was chosen for the infection studies since, in this mutant, the Ail/OmpX, OmpA, and Pla surface antigens would be more fully exposed to neutralizing antibodies. All of the unimmunized, naive control animals died by day 20, although 90% of the animals were dead by day 10. Similarly, antibodies to Pla did not provide any protection to mice (data not shown). In sharp contrast, antibodies to both OmpA and Ail/OmpX provided protection to mice resulting in 40 to 50% animal survival, respectively. In fact, Ail/OmpX- and OmpA-immunized mouse survival curves were significantly different than unimmunized mouse survival curves ($P = 0.001$ to 0.0066) (Fig. 6B).
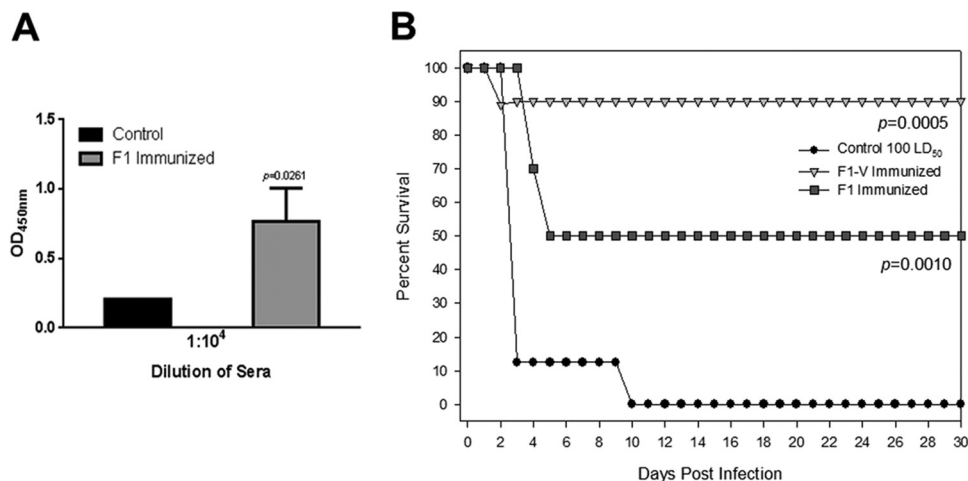

FIG 5 Active immunization of mice with F1 and LcrV provided protection to animals against pneumonic plague when challenged by the i.n. route with 100 LD_{50} of WT CO92. Antibody titers to F1 antigen in the mouse sera compared to that of preimmune sera (control) are shown in panel A, while animal protection data are shown in panel B. The data were statistically analyzed using Kaplan-Meier survival estimates, and the *P* values are presented.
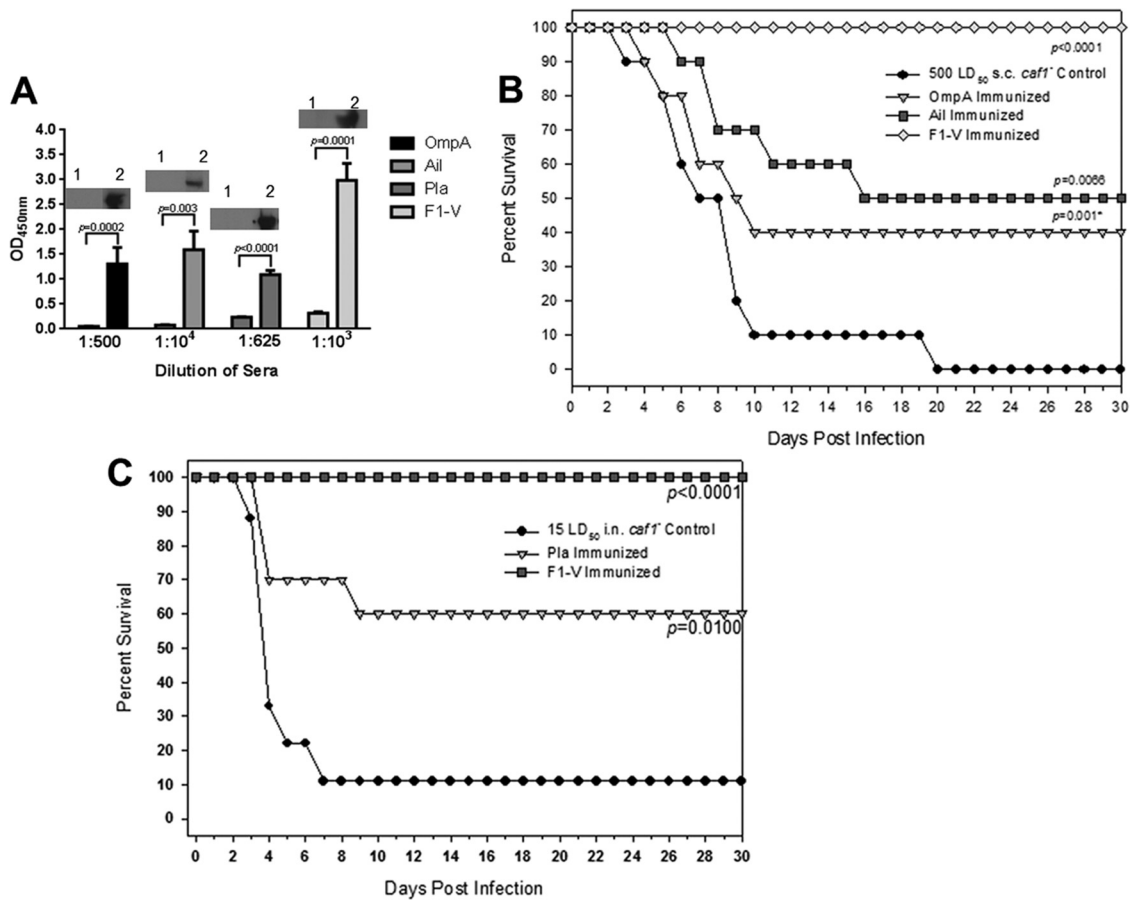
FIG 6 (A) Evaluation of antibody titers in the sera of mice after active immunization with OmpA, Ail, Pla, and F1-V proteins. The individual animal serum was titrated in duplicate after serial 5- or 10-fold dilution. Preimmune sera were used as controls. The insets show the detection of the purified antigen using specific serum to a particular antigen (lane 1, by using preimmune serum; lane 2, by using postimmunization serum). (B) Active immunization of mice with F1-V, OmpA, and Ail provided protection to mice against developing bubonic plague. Animals were challenged with the $\Delta caf1$ mutant at the indicated dose. (C) Pla-immunized mice were 60% protected against i.n. challenge of $\Delta caf1$ mutant at a 15 LD$_{50}$. The data were statistically analyzed by using Kaplan-Meier survival estimates, and the P values are presented. *, P when the data were analyzed using an unpaired Student t test. This value was 0.08 with the Kaplan-Meier survival estimates.

F1-V immunization clearly provided the greatest degree of protection whereby 100% of immunized mice survived s.c. challenge ($P < 0.0001$) (Fig. 6B). In one group, we immunized mice with all three antigens Ail/OmpX, OmpA, and Pla; there was no additive or synergistic effect on protection against bubonic plague. In fact, the protective effects of antibodies to three antigens in cocktail were similar to that noted for Ail/OmpX alone (data not shown).

Interestingly, in a pneumonic plague mouse model, only antibodies to Pla provided protection to animals when challenged with 15 LD$_{50}$ (7,500 CFU) of $\Delta caf1$ mutant of CO92 (Fig. 6C) despite Pla immunization not protecting against a bubonic plague challenge (data not shown). At this dose, while 90% of the unimmunized mice died by day 9, 60% of the Pla-immunized animals were protected from developing pneumonic plague ($P = 0.01$) (Fig. 6C). Neither Ail/OmpX nor OmpA immunizations protected mice from developing pneumonic plague (data not shown) despite immunizations with both of the aforementioned antigens providing protection against bubonic plague in mice (Fig. 6B). We have shown earlier (39) that the LD$_{50}$ of WT CO92 and its $caf1$ mutant were essentially similar in a mouse model of pneumonic plague.

LD$_{50}$ determination of WT *Y. pestis* CO92 in a rat model of bubonic and pneumonic plague. In addition to testing the efficacy of Ail/OmpX, OmpA, and Pla putative vaccine candidates in mice (which we routinely do) (42), we also sought to employ a rat model of infection, as we have done in the past (43), for comparative purposes. However, prior to undergoing immunization (with our three candidate antigens) and challenge studies, we first determined the LD$_{50}$ of the WT *Y. pestis* CO92 for both bubonic and pneumonic plague, so we conducted a dose-response experiment in a rat model ($n = 5$/group). The LD$_{50}$s in rats by the i.n. and s.c. routes were calculated to be 500 and 100 CFU, respectively, for WT CO92. Therefore, for rat challenge studies, we used 8 to 10 LD$_{50}$ (4.0×10^3 to 5.0×10^3 CFU) and 8 LD$_{50}$ (8.0×10^2 CFU) for evoking pneumonic and bubonic plague, respectively, which typically cause 100% mortality in animals between 3 and 8 days depending upon the route of infection. As shown in Fig. 7, both of the aforementioned routes of infection resulted in 0% rat survival by day 8 p.i. when infected with 1×10^3 CFU (i.n.) and 4×10^3 CFU (s.c.).

For evaluating the $\Delta caf1$ mutant in rats, we used two i.n. challenge doses of 2.75×10^6 CFU and 1.65×10^7 CFU, which corresponded to WT equivalent of 5,500 and 33,000 LD$_{50}$s., respec-

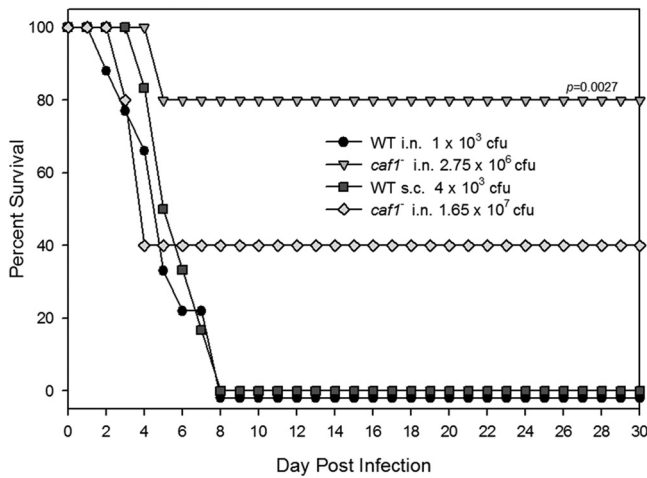FIG 7 Evaluation of the Δ*caf1* mutant and its corresponding WT *Y. pestis* CO92 in the rat model of pneumonic and bubonic plague. The animals were infected either via the i.n. or s.c. route at the indicated doses. Rat survival was recorded over a period of 30 days. The data were statistically analyzed by using Kaplan-Meier survival estimates, and the *P* values are presented.

tively. At the lower challenge dose (2.75×10^6 CFU), 80% of infected mice survived out to 30 days p.i. ($P = 0.0027$ relative to the WT i.n. challenge rat survival), while the higher dose (1.65×10^7 CFU) resulted in as high as 40% rat survival (Fig. 7). Unlike in mice (Fig. 6C), the Δ*caf1* mutant was highly attenuated in the rat i.n. challenge model of infection, and, as a result, we used the WT CO92 *Y. pestis* strain in our rat studies when testing the efficacy of Ail/OmpX, OmpA, and Pla as protective antigens.

Protective effects of antibodies to Ail, OmpA, and Pla during bubonic and pneumonic plague in a rat model. As we had previously reported for our mouse studies (Fig. 6A), prior to carrying out animal survival experiments on Pla-, OmpA-, or Ail/OmpX-immunized rats, we first sought to demonstrate whether humoral responses against the aforementioned antigens were being generated in rats. Indeed, we were able to demonstrate somewhat com-

parable antibody titers in rats to all three studied antigens in addition to F1-V (Fig. 8A). Using the *Y. pestis* CO92 WT strain (as opposed to the Δ*caf1* mutant) in our rat challenges, for reasons described above, we determined that whereas antibodies to Ail/OmpX were protective against developing pneumonic plague ($P = 0.0268$) at a LD_{50} of 8.6 (Fig. 8B), anti-OmpA and anti-Pla antibodies failed to provide any protection (data not shown). Our F1-V subunit vaccine resulted in 100% rat protection out to day 30 p.i. ($P < 0.0001$) (Fig. 8B). In a bubonic plague model, none of the antibodies (to Ail/OmpX, Pla, or OmpA) provided any protection to rats (at 7 LD_{50}).

DISCUSSION

Much of the emphasis on plague vaccine development has centered on F1 and LcrV antigens, particularly in subunit vaccine research (see reference 3 and references therein). However, we argue that focusing exclusively on F1 and LcrV as the sole protective antigens suitable for exploitation as vaccine candidates is shortsighted. This is so on account of natural variation in the *lcrV* open reading frames of various *Y. pestis* isolates (7, 8, 44), as well as on account of the existence of highly virulent *Y. pestis* isolates that lack the F1 antigen altogether (7, 8). Taken together, the search for additional protective antigens should continue and was the basis of the present study.

To directly address this, we sought to identify novel protective *Y. pestis* antigens and determined their effectiveness as putative vaccine candidates against both pneumonic and bubonic mouse and rat models of infection. Both routes of infection were studied since disease outbreaks could occur via flea bite or inhalational, either naturally or intentionally, and we wanted to develop a more comprehensive and holistic assessment of the protective nature of our antigens in question. In this study, for example, Pla did not protect mice against bubonic plague but did confer protection against the pneumonic form of the disease, while the converse was true for the Ail/OmpX and OmpA antigens.

In that same vein, we used both mouse and rat models of infection since we were one of the first groups to use rats to study pneumonic plague pathogenesis (43) and have since learned that
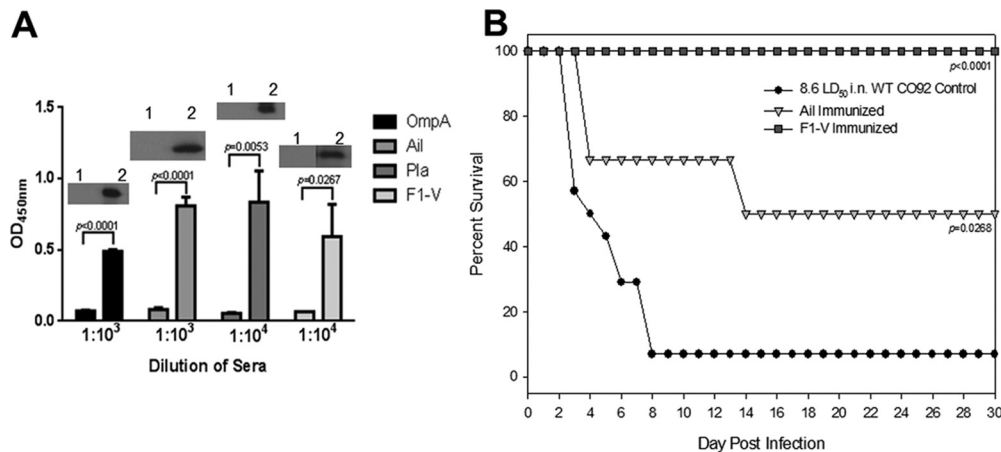

FIG 8 (A) Evaluation of antibody titers in the sera of rats after active immunization with OmpA, Ail, Pla, and F1-V proteins. Individual animal sera were titrated in duplicate after serial 10-fold dilution. Preimmune sera were used as controls. The insets show the detection of the purified antigen using specific serum to a particular antigen (lane 1, by using preimmune serum; lane 2, by using postimmunization serum). (B) Active immunization of rats with F1-V and Ail provided protection to animals against developing pneumonic plague. The animals were challenged with the WT CO92 at a LD_{50} of 8.6. The data were statistically analyzed by using Kaplan-Meier survival estimates, and the *P* values are presented.

each model can reveal data that the other model does not (42, 43). In support of this statement, we presented (in this report) that mice and rats did not generate the same protective immune responses against *Y. pestis* infection in levofloxacin-rescued animals. Furthermore, unlike in mice (39), the Δ*caf1* mutant was highly attenuated in the rat i.n. model of infection as shown in this study. Indeed, we, and other investigators, have shown that F1 does not contribute to *Y. pestis* pathogenesis during pneumonic plague but does participate in contributing to virulence during bubonic plague in mice (38, 45). However, vaccines composed of either F1 or F1 and V antigens, as well as the V-F1 fusion protein contribute to developing protection against pneumonic plague in nonhuman primates (6, 46, 47). In earlier studies, protection was shown in a mouse model against both bubonic and pneumonic plague by passive immunization with monoclonal antibodies to F1 (48) and the recombinant F1-V antigen fusion vaccine (12). These data indicated that immunoprotective antigens need not be virulence factors for the bacterium. As a result, we had to use the WT CO92 *Y. pestis* strain in our rat studies when testing the efficacy of our putative Ail/OmpX, OmpA, and Pla subunit vaccines.

Only Ail/OmpX protected rats against pneumonic plague, while antibodies to other antigens were not protective against bubonic and pneumonic plague in rats. These findings serve to underscore the importance of using multiple infection model systems when determining the efficacy of novel vaccine candidates. Whether these differences were related to different subclasses of antibodies produced or activation of different subsets of T cells needs to be further explored as a future study. Based on the data presented in Fig. 1B, it is apparent that sera from *Y. pestis*-infected and levofloxacin-rescued mice did not have similar *Y. pestis* antigen-specific antibodies that were observed in similarly infected and treated rats.

It has been reported that the genetic makeup of the host dictates susceptibility or resistance to *Y. pestis* infection. For example, C57BL/6J mice were shown to be more susceptible to developing bubonic plague than BALB/cJ mice by the Δ*caf1* mutant strain of *Y. pestis* (17). Likewise, C57BL/6J and BALB/c substrains, such as BALB/cByJ and BALB/cAnNHsd, were highly susceptible to infection with the *Y. pestis* KIM5 strain compared to the BALB/cJ mouse strain exhibiting 250-fold-higher LD_{50}s for KIM5 (49). This resistance of BALB/cJ mice to *Y. pestis* infection correlated well with the presence of a plague resistance locus (*prl1*) that maps to the major histocompatibility region on chromosome 17, which carries innate and adaptive immunity-associated genes (49). Therefore, subtle differences in the immune responses of mice and rats to *Y. pestis* infection and the much higher LD_{50} of the Δ*caf1* mutant in a rat model of pneumonic plague compared to the WT *Y. pestis* CO92 could be genetically linked to specific rodent species and requires further evaluation.

Finally, since antigens were purified under denaturing conditions, this could have impacted the level of protection noted in animals. Since antibodies to conformational epitopes to these antigens could be important, we will explore this possibility by expressing these antigen-encoding genes in different expression vector systems, which could provide us with soluble antigens.

Interestingly, all four of the protein targets identified by our hyperimmune Western blot screen, have been previously designated as either bona fide virulence factors or as virulence-associated factors. When considering the collective literature demonstrating the importance of Pla, OmpA, and Ail/OmpX in *Y. pestis*'s pathogenesis, the fact that our hyperimmune Western blot screen identified these three outer membrane proteins (in addition to F1) becomes less surprising. However, what remained unknown was whether any of these outer membrane antigens could serve as useful subunit vaccine candidates.

In the present study, we made considerable progress in identifying novel vaccine candidate antigens for a potential *Y. pestis* subunit vaccine. Such a vaccine candidate could use one, two, or even all three of our candidates in cocktail either alone or in addition to F1-V. This is particularly important since active immunization of cynomolgus macaques with F1-V provided significant protection against pneumonic plague, while African green monkeys were not well protected against developing pneumonic plague (7). Therefore, a cocktail of vaccine antigens may provide optimal protection against different forms of plague in multiple models.

Overall, based on our data, antibodies to Ail/OmpX and OmpA provided protection to mice against bubonic but not pneumonic plague and anti-Pla antibodies were protective against pneumonic but not bubonic plague. In a rat model, anti-Ail/OmpX antibodies were protective against developing pneumonic plague. The immunological basis for this variation is unknown at this time, but this reinforces the need to test new immunogens in multiple animal model systems to fully elucidate their potential as important vaccine components. Indeed, it has been reported that while F/LcrV-based vaccines are protective in cynomolgus macaques but the protection level was highly variable (between 0 and 75%) against pneumonic plague in African green monkeys (50). Whether such differences could be pivotal in providing protection against bubonic versus pneumonic plague in different animal models represent one scenario which needs to be further investigated.

Finally, although antibodies to F1-V provided 100% protection to animals against plague when infected by the WT *Y. pestis* CO92, this protection is expected to be much lower when infection occurs with the F1⁻ strains of *Y. pestis* or those *Y. pestis* strains which have different variants of LcrV. In such scenarios, addition of other antigens, such as Ail/OmpX, Pla, and/or OmpA could be beneficial as antibodies against them would certainly help to negate severe plague infections in vaccinated people. This would allow widening the window for other treatment options, such as antibiotics to save plague-infected individuals.

Although our initial study indicated that immunization of mice with equal amounts of Ail/OmpX, OmpA, and Pla did not provide any added benefit (in terms of animal protection), different ratios of these antigens must be tested, and any competition among these antigens in producing protective antibodies should be explored. Indeed, F1 and LcrV antigens must be used in a specific ratio during immunization to demonstrate optimal protection in animals (5, 6, 47). We were able to demonstrate that OmpA, Pla, and Ail/OmpX were all protective in either bubonic or pneumonic *Y. pestis* challenges in either mouse or rat models of infection. It will be important in the future to evaluate whether the addition of the above-mentioned antigens to an F1-V subunit vaccine could provide enhanced protection against *Y. pestis* strains that are devoid of the capsule or carry different variants of LcrV. Such a multivalent vaccine could generate both a robust and broad adaptive immune response targeting multiple surface-exposed *Y. pestis* antigens. Taking such an approach could yield the most efficacious *Y. pestis* vaccine to date.

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