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Yersinia pestis IS*1541* Transposition Provides for Escape from Plague Immunity▽

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***Yersinia pestis* is perhaps the most feared infectious agent due to its ability to cause epidemic outbreaks of plague disease in animals and humans with high mortality. Plague infections elicit strong humoral immune responses against the capsular antigen (fraction 1 [F1]) of *Y. pestis*, and F1-specific antibodies provide protective immunity. Here we asked whether *Y. pestis* generates mutations that enable bacterial escape from protective immunity and isolated a variant with an IS*1541* insertion in *caf1A* encoding the F1 outer membrane usher. The *caf1A*::IS*1541* insertion prevented assembly of F1 pili and provided escape from plague immunity via F1-specific antibodies without a reduction in virulence in mouse models of bubonic or pneumonic plague. F1-specific antibodies interfere with *Y. pestis* type III transport of effector proteins into host cells, an inhibitory effect that was overcome by the *caf1A*::IS*1541* insertion. These findings suggest a model in which IS*1541* insertion into *caf1A* provides for reversible changes in envelope structure, enabling *Y. pestis* to escape from adaptive immune responses and plague immunity.**

Yersinia pestis is the causative agent of plague, a devastating disease that in three pandemics resulted in more human deaths than any other infectious agent has (45, 69). Depending on the mode of transmission (flea bite or aerosol droplets), *Y. pestis* can precipitate bubonic or pneumonic plague infections (35, 53). High mortality and epidemic spread of plague, together with the possibility of weaponized use, have been the impetus for the development of plague vaccines (55, 59). Both whole-cell killed and live attenuated plague vaccines are efficacious (23, 30, 38, 56); however, due to the serious side effects of whole-cell preparations, vaccine development has shifted toward purified subunits (39, 55). Protective immunity following bubonic plague infection or immunization with live attenuated vaccine strains is based on humoral immune responses against capsular antigen, which is assembled as a layer of F1 pili (10, 48, 51). Located on the fraction I plasmid pFra (20, 36), the *caf1* structural gene for F1 pili is part of an operon with *caf1M* and *caf1A* encoding the periplasmic chaperone Caf1M (19, 70) and the outer membrane usher of pilus assembly Caf1A, respectively (51) (Fig. 1). Caf1R, the transcriptional regulator of the *caf1A1M1* operon, is encoded by a divergently transcribed gene on pFra (29). F1 vaccines elicit protective immunity when they are used either alone (2, 4) or in combination with a second protective antigen, LcrV (13, 24, 28, 66). In contrast to LcrV, which is deposited at the tip of type III needle complexes (41) and is essential for *Y. pestis* type III injection into host cells and virulence (34, 40, 46, 47), expression of *caf1* is dispensable for plague pathogenesis in mice, guinea pigs, and nonhuman primates (13, 48, 68). Recently, Sebbane and colleagues discovered that the expression of F1 pili contributes to the efficient transmission of plague by infected *Xenopsylla cheopis* fleas that feed on mice (52).

An F1 mutant, nonencapsulated plague strain was isolated from autopsy samples from a human plague victim (67), and naturally occurring or genetically engineered F1 variants are thought to cause animal plague infections (7, 13, 18, 63–65). Here, we address the possibility that there are different types of breakthrough variants in animals immunized with live attenuated whole-cell vaccines or F1-based subunit vaccines and pursue their molecular analysis. We report that insertion of a mobile genetic element into the *caf1A* structural gene for F1 pilus (capsular) assembly is an escape mechanism of *Y. pestis* for naturally occurring immunity in infected animals.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Y. pestis* CO92, a fully virulent wild-type plague isolate (16), was grown on heart infusion agar (HIA) supplemented with 0.2% galactose and 0.0075% Congo red (58) and incubated at 26°C for 48 h. The mean lethal dose (MLD) for bubonic plague following subcutaneous injection into BALB/c mice is 1 CFU for *Y. pestis* CO92. *Y. pestis* KIM D27 (pFra$^+$, pCD1$^+$, pPCP1$^+$, Δ*pgm*) (6) was used as the parental strain for allelic replacement. The KIM D27 ΔF1 strain has been described previously (48). The KIM D27 *caf1A*::IS*1541* (CAC2) mutation was generated by amplifying the *caf1A* gene from plasmid pFra of strain CO92 *caf1A*::IS*1541* (CAC1) with the following primers: caf1AFOSphI (5′-TAGCATGCATGAGGTATTCAAAGCTGTTCC-3′) and caf1ADOSacI (5′-TAGAGCTCTCAGTTATTTAAGATGCAGG-3′). The amplified product was ligated into the SphI/SacI sites of pCVD442 (Amp^r). The recombinant plasmid was electroporated into KIM D27, and single-crossover events were selected by plating bacteria on HIA supplemented with 50 μg/ml ampicillin. Resolution of replication-defective plasmid cointegrates was achieved by plating bacteria on HIA supplemented with 10% sucrose for counterselection for *sacB* (5). Ampicillin-sensitive and sucrose-resistant colonies were examined by PCR and DNA sequencing to confirm mutant genotypes. We screened 250 colonies with this technology and identified one mutant, which was designated CAC2.

To assess IS*1541* transposition into *caf1A* in vitro, *Y. pestis* KIM D27 was grown in 20 ml of heart infusion broth (HIB) with 2.5 mM $CaCl_2$ overnight at 37°C. Bacterial dilutions were plated on HIA and incubated overnight at 37°C. Ten thousand colonies were lifted using nitrocellulose filters (Protran BA5; Schleicher & Schuell) and immunoblotted with monoclonal antibody (MAb) F1-04-A-G1. All colonies examined displayed the F1$^+$ phenotype.

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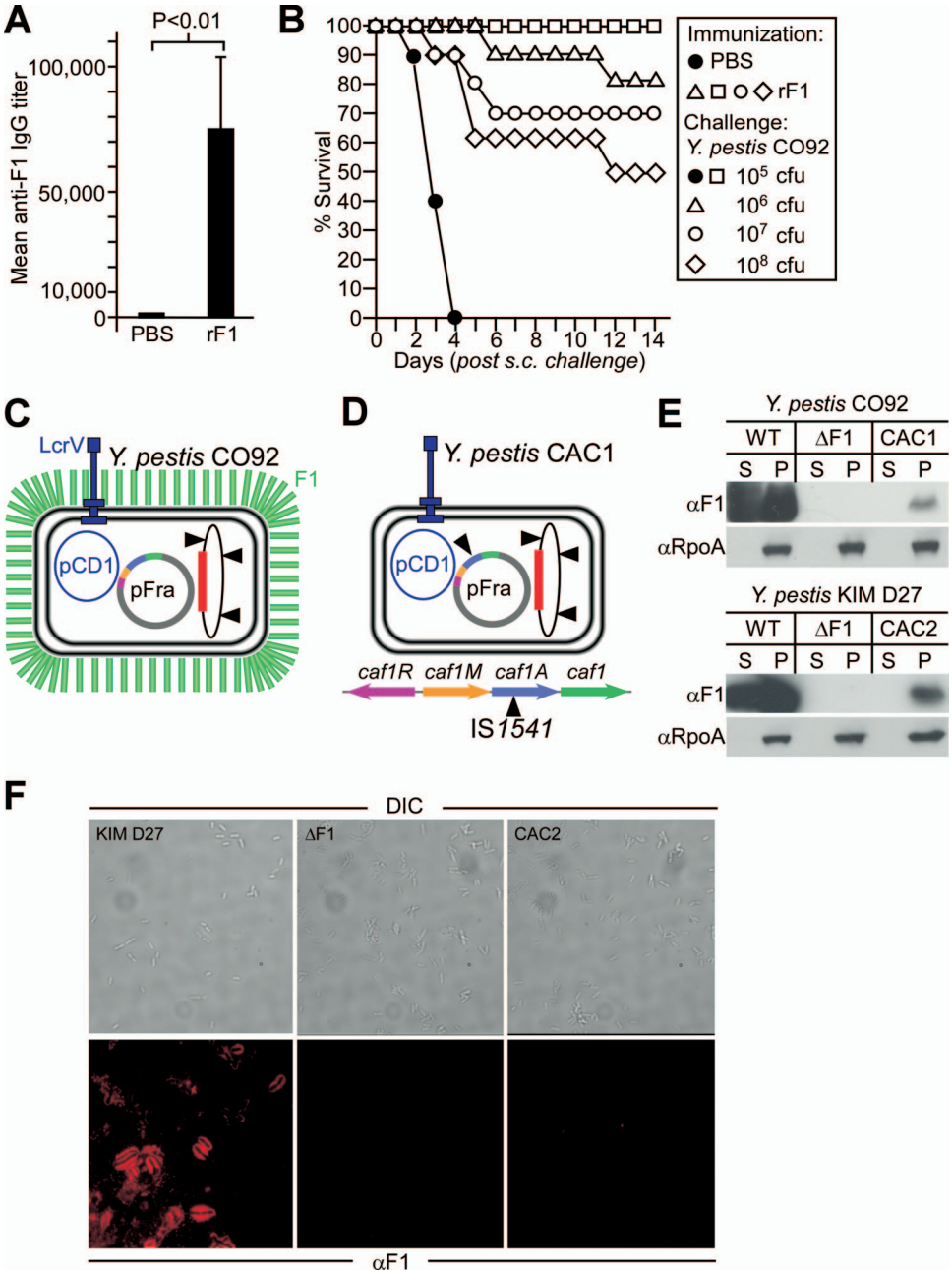

FIG. 1. Isolation of *Y. pestis* escape variants. (A) Mice were immunized with recombinant capsular antigen (rF1) or a PBS control, and serum IgG antibody titers were determined by ELISA. (B) rF1-immunized mice (cohorts of 10 animals) were challenged with increasing doses of wild-type strain *Y. pestis* CO92, and their survival was monitored. (C) Diagram showing *Y. pestis* CO92 and its virulence plasmids pCD1 (providing LcrV and type III secretion [blue]) and pFra (with the *caf1R* gene and the *caf1M-caf1A-caf1* operon for biogenesis of F1 pili [green]), as well as the chromosome with a high-pathogenicity island–pigmentation segment (*pgm*) (red) and IS*1541* elements (arrowheads). (D) Insertion of IS*1541* into *caf1A* abrogates F1 secretion and capsule assembly. (E) Cultures of *Y. pestis* CO92, KIM D27 (Δ*pgm*), and isogenic variants with a *caf1* deletion (ΔF1) or *caf1A*::IS*1541* (CAC1 and CAC2) were centrifuged, and proteins in the culture supernatant (S) and bacterial pellet (P) were analyzed by immunoblotting with F1-specific antibody (αF1) and RNA polymerase-specific antibody (αRpoA). (F) *Y. pestis* strains were analyzed by differential interference contrast (DIC) or fluorescence microscopy with F1-specific antibody. s.c., subcutaneous; WT, wild type.

Protein analysis. Proteins were separated by electrophoresis on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with Coomassie brilliant blue. For immunoblotting, proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed with antisera as previously described (11).

Digitonin fractionation assay. HeLa cells were grown in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum and 2 mM glutamine at 37°C in the presence of 5% CO_2. Overnight *Y. pestis* cultures in HIB were grown at 26°C. The following day, cultures were diluted 1:20 into fresh HIB containing 2.5 mM $CaCl_2$ with an appropriate antibiotic and were grown at 26°C for 2 h. One hour prior to infection, 90% confluent HeLa cells were washed two times with phosphate-buffered saline (PBS) and 10 ml of Dulbecco modified Eagle medium (GIBCO). HeLa cells were infected with the *Yersinia* strains indicated below at a multiplicity of infection of 10. Three hours following infection, the tissue culture medium was decanted and centrifuged at 15,000 rpm for

15 min. Proteins in 10 ml of the supernatant were precipitated with trichloroacetic acid, and the rest of the supernatant was discarded. The pellets were suspended in 10 ml of 1% SDS in PBS, and 10 ml of the pellet fraction was precipitated with trichloroacetic acid. Digitonin (1% in PBS) was added to the tissue culture cells and their attached bacteria and incubated for 20 min at room temperature to disrupt HeLa cell plasma membranes. Following HeLa cell disruption, cell remnants were detached from the flasks using a cell scraper, digitonin lysates were centrifuged, and proteins in the supernatant and pellet were processed as described above (33). Samples were separated on 15% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted.

Cytotoxicity assay. HeLa cell tissue cultures (2×10^5 cells) were grown in 12-well tissue culture plates with OptiMEM. Six hours prior to infection, overnight *Yersinia* cultures were diluted 1:20 into fresh HIB containing 5 mM $CaCl_2$ with an appropriate antibiotic. The cultures were grown at 26°C for 1 h and then shifted to 37°C for 5 h. HeLa cells were infected with *Y. pestis*, which had been premixed for 25 min at room temperature with 500 μg MAb F1-04-A-G1 (USAMRIID) or a similar volume of 1× PBS, at a multiplicity of infection of 10 and incubated for 3 h at 37°C in the presence of 5% CO_2. Cells were washed with PBS and fixed with 3.7% formaldehyde for 20 min. Fixation was quenched with 0.1 M glycine in PBS for 5 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min at 4°C. Cells were washed and blocked for 15 min with PBS containing 0.05% Tween 20 and for 24 h with 5% skim milk. Actin filaments were labeled with 99 nM (3 U) of rhodamine-phalloidin (Flexa) for 20 min at room temperature. The labeling solution was removed, and each well was washed with PBS. Cells were visualized with a Nikon TE-2000-U inverted microscope. Rhodamine visualization was achieved using excitation at 591 nm and emission at 608 nm. Images were captured with a Cascade 1K charge-coupled device camera.

Purification of rLcrV, rV10, and rF1. The pET16b (Novagen) expression vectors (57) for recombinant LcrV (rLcrV), recombinant V10 (rV10), and recombinant F1 (rF1) have been described previously (48). Briefly, cultures of *Escherichia coli* BL21(DE3) carrying the expression vectors were grown overnight at 37°C in Luria-Bertani medium with 100 μg/ml ampicillin. Bacteria were diluted in fresh medium and grown to an optical density at 600 nm of 0.5. T7 polymerase was induced with 1 mM isopropyl-1-thiol-D-galactopyranoside, and bacterial growth was continued for 3 h at 37°C. Bacteria were sedimented by centrifugation at $10{,}000 \times g$ for 15 min, and *E. coli* cells from a 500-ml culture were disrupted twice in a French pressure cell at 14,000 lb/in^2 in 20 ml of 50 mM Tris-HCl (pH 7.5)-150 mM NaCl (column buffer). Lysates were applied to a nickel nitrilotriacetic acid column (bed volume, 1 ml) preequilibrated with 20 ml column buffer. The column was washed with 20 volumes of the same buffer and then with 20 volumes of column buffer containing 20 mM imidazole. Protein was eluted in 50 mM Tris-HCl (pH 7.5)-150 mM NaCl with 250 mM imidazole. Proteins were extracted with Triton X-114 (Sigma) to remove endotoxin, and the detergent was removed by chromatography on a HiTrap desalting column (GE); proteins were eluted in PBS. Lipopolysaccharide contamination of vaccine antigens was assayed with *Limulus* amebocyte lysate (QCL-1000; Cambrex, New Jersey). Protein concentrations were determined with the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) or by measuring absorption at 280 nm. Proteins were flash frozen with dry ice and ethanol and stored at −80°C until they were used.

Immunization of animals. Groups of 6- to 8-week-old female BALB/c mice (Charles River Labs, Massachusetts) were immunized twice by intramuscular injection into the hind leg with 0.1-ml aliquots containing 50 μg of rLcrV, rV10, rF1, rLcrV plus rF1, or rV10 plus rF1 in 25% Alhydrogel (Brenntag Biosector, Frederikssund, Denmark) on day 0 and day 21. Blood was drawn on day 35 to measure serum antibody titers prior to plague challenge. Attenuated *Y. pestis* strains were grown overnight in HIB at 26°C, diluted 1:100 into fresh media, and grown for 3 h at 26°C. Bacteria in each culture were sedimented by centrifugation, washed, and diluted in PBS to obtain the required concentration. Groups of 6- to 8-week-old female BALB/c mice (Charles River Labs, Massachusetts) were immunized by intramuscular injection into the hind leg with 0.1-ml aliquots containing 1×10^5 CFU of *Y. pestis* KIM D27 suspended in PBS. Following injection, mice were monitored for 21 days. Blood sampling and challenge occurred at day 21.

Plague challenge of immunized animals. Two mouse models were used to recapitulate the pathogenesis of bubonic plague (subcutaneous injection) and pneumonic plague (intranasal instillation). For the pneumonic plague model, mice were anesthetized with 17 mg/ml ketamine (Ketsed; Vedco) and 0.7 mg/ml of xylazine (Sigma) injected into the peritoneal cavity and then were challenged by intranasal inoculation with 20 μl of a *Y. pestis* suspension in PBS (14, 48). For this experiment, *Y. pestis* CO92 or CAC1 was grown in HIB supplemented with 2.5 mM calcium at 37°C overnight. Bacteria were washed and diluted in sterile PBS to obtain the required concentration. For passive transfer experiments and bubonic plague challenge, mice were inoculated with 200 μl of mouse serum 1 h prior to challenge by subcutaneous injection with a 0.1-ml suspension containing 10 MLD of *Y. pestis* CO92 or CAC1; for this experiment, plague bacteria were grown in HIB at 26°C overnight, washed, and diluted in sterile PBS to obtain the required concentration. For the breakthrough experiment, the mice were challenged with 0.1-ml aliquots containing 10^5 to 10^8 MLD of *Y. pestis* CO92 (1×10^5 to 1×10^8 CFU) subcutaneously. Immunized mice that had been challenged with *Y. pestis* CO92 were monitored for morbidity and mortality for 14 days. All mouse experiments were performed in accordance with institutional guidelines following review of the experimental protocol and approval by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Chicago. All animal experiments were performed in collaboration with the GLRCE Animal Research & Immunology Core at the University of Chicago. When possible and appropriate, plague-infected moribund animals were killed by asphyxia followed by cervical dislocation prior to necropsy, microbiology, or histopathology analyses. The Mann-Whitney test was used for statistical analysis of antibody titers. Fisher's exact test was used for statistical analysis of animal mortality following plague challenge.

Histopathology. Animal tissues obtained during necropsy were fixed in 10% neutral buffered formalin and embedded in paraffin. Blocks were sectioned (5-μm sections) and stained with hematoxylin and eosin prior to microscopy and image analysis.

Antibody detection. Levels of serum immunoglobulin G (IgG) with specific antigen binding activity were determined by a custom enzyme-linked immunosorbent assay (ELISA) at the GLRCE Animal Research & Immunology Core at the University of Chicago (43).

Phagocytosis. Blood was obtained from human volunteers by vein puncture. One-milliliter samples were anticoagulated with lepirudin (Refludan) and infected with 1×10^5 CFU *Y. pestis* in the presence or absence of 0.1-ml serum samples. At timed intervals, the survival of plague bacteria was determined using aliquots spread on agar plates and incubated for colony formation. Experiments with human volunteers involved protocols that were reviewed by, approved by, and performed under regulatory supervision of the University of Chicago's Institutional Review Board.

Immunofluorescence microscopy. *Y. pestis* KIM D27, ΔF1, and CAC2 were grown in 4 ml HIB with 2.5 mM $CaCl_2$ overnight at 37°C, and bacteria in the cultures were sedimented by centrifugation (5 min at $6{,}000 \times g$). Bacteria in the sediment were washed with 1 ml of PBS (10 mM sodium phosphate), fixed with 2.5% paraformaldehyde and 0.006% glutaraldehyde in 30 mM PBS (pH 7.4) for 20 min at room temperature, and washed three times with 1 ml PBS. Bacterial suspensions (30 μl) were applied to L-polylysine-coated coverslips for 5 min, washed three times with 60 μl PBS to remove nonadherent cells, and allowed to dry. Plague bacteria were rehydrated in 60 μl of PBS for 5 min, blocked with 3% bovine serum albumin in PBS for 45 min, and then incubated for 1 h with purified anti-F1 polyclonal rabbit serum in 3% bovine serum albumin in PBS. The anti-F1 polyclonal rabbit serum was purified by incubating the serum for 2 h with acetone-precipitated antigen derived from whole-cell preparations of strain *Y. pestis* ΔF1. Purified serum was used at a final concentration of 1:1,000. Bacteria were washed 10 times with 100 μl PBS and incubated for 1 h in the dark with Alexa Fluor 647 goat anti-rabbit IgG (1:200; Invitrogen). Cells were washed 15 times with 60 μl PBS, and slides were prepared for microscopy and viewed with a Leica SP5 AOBS spectral 2-photon confocal microscope or a Leica DMI6000 inverted microscope with conventional fluorescence (100 W Hg) and differential interference contrast optics using a 63× oil objective (NA 1.4) with automatically optimized confocal pinhole apertures. Images were captured with a chilled photomultiplier tube fluorescence detector (with digital spectral definition in 1-nm increments) and one transmitted light detector with 12-bit output and 6.5× and 13.5× digital zoom. Captured images were analyzed with Image J software.

Nucleotide sequence accession number. The annotated sequence of the IS*1541* insertion in the *caf1A* gene of *Y. pestis* CAC1 has been deposited in the GenBank database under accession number FJ687152.

RESULTS

***Y. pestis* breakthrough challenge of immunized mice.** To examine *Y. pestis* escape from F1-mediated immune responses, we purified rF1 and immunized cohorts of BALB/c mice using two intramuscular injections of 50 μg rF1 adsorbed to Alhydrogel separated by a 21-day interval. Vaccine success was measured by using antibody titers in serum samples (Fig. 1A).

TABLE 1. Plague bacteria isolated from rF1-immunized animals

Animal[a]	Challenge dose (CFU)[b]	Time of death (days)[c]	CFU in tissue homogenates at necropsy				PCR (*lcrV* and *caf1*)[d]	No. of escape survivors/no. challenged[e]
			Lymph node	Spleen	Liver	Blood		
1	10^7	5	2×10^6	0	0	0	+/+	10/10
2	10^7	6	1×10^8	1×10^5	0	0	+/+	1/10
3	10^8	5	0	0	0	0	NA[f]	
4	10^8	12	3×10^7	8×10^6	2×10^7	2×10^4	+/+	10/10

[a] Animals used for the Kaplan-Meyer analysis (Fig. 1B).
[b] *Y. pestis* CO92 suspended in 100 μl PBS was injected subcutaneously.
[c] Time of death following injection of the challenge dose.
[d] The results of PCR tests with primers specific for *lcrV* located in pCD1 and *caf1* located in pFra were scored as positive or negative.
[e] Number of rF1-immunized animals (average anti-F1 IgG ELISA titer, 1:75,000) that survived challenge with 1,000 CFU of plague bacteria isolated during necropsy of animals/number challenged.
[f] NA, not applicable.

On average, immunized animals harbored rF1-specific IgG with titers of 1:75,000 (±31,000), whereas mock-immunized animals displayed titers of <1:800 ($P < 0.01$). Mice were challenged by subcutaneous injection with increasing doses of the highly virulent wild-type isolate *Y. pestis* CO92. Immunized animals challenged with 1×10^5 MLD were fully protected from lethal bubonic plague, whereas animals that received adjuvant alone (PBS and Alhydrogel) succumbed to the disease (Fig. 1B). Animals challenged with 1×10^6 MLD exhibited 20% mortality. The frequency of lethal disease increased when the challenge dose was increased to 1×10^7 and 1×10^8 MLD (Fig. 1B). Lymph node, blood, lung, and spleen tissue homogenates for two animals from each of the high-challenge-dose cohorts were spread on Congo red agar plates, and isolated colonies were analyzed by PCR (Table 1). Bacterial isolates from rF1-immunized animals exhibited positive PCR tests for *caf1* and *lcrV*. F1-immunized animals challenged with 1×10^7 MLD harbored plague bacteria in their lymph nodes, and in one animal the bacteria had spread to the spleen. One of the animals challenged with 1×10^8 MLD had a disseminated infection (Table 1). As a test for escape variants, one colony each of the isolated strains was reinoculated into rF1-immunized animals. Mice infected with the isolate from animal 2 succumbed to this challenge (Table 1).

***Y. pestis* breakthrough variants harbor an IS*1541* insertion in *caf1A*.** *Y. pestis* CAC1 (isolated from animal 2) failed to secrete F1; however, small amounts of pilus antigen were found in the bacterial sediment (Fig. 1E). PCR and DNA sequencing revealed the absence of mutational lesions in *caf1*; however, the *caf1A* gene harbored an IS*1541* insertion in reverse orientation at nucleotide 1263 of its open reading frame, which disrupted the outer membrane usher and *Y. pestis* pilus assembly (51) (Fig. 1C and D). To test whether the *caf1A*::IS*1541* lesion is solely responsible for the defect in pilus assembly, the mutation was introduced into the nonpigmented variant *Y. pestis* KIM D27 (6) to generate strain CAC2 (Fig. 1E). Fractionation of bacterial cultures confirmed that the *caf1A*::IS*1541* mutation indeed abolished F1 secretion. When viewed using microscopy and fluorescent antibody, *Y. pestis* KIM D27 displayed an F1 capsule, whereas isogenic *caf1* deletion and *caf1A*::IS*1541* insertion variants did not (Fig. 1F).

***Y. pestis caf1A*::IS*1541* variants are fully virulent.** To examine the virulence of the *caf1A*::IS*1541* variants in naïve animals, BALB/c mice ($n = 10$) were infected by subcutaneous injection with increasing doses *Y. pestis* CAC1 (10, 20, and 100 CFU) or with CO92 (10 CFU) (Fig. 2A). While the growth rates of the CAC1 and CO92 strains in laboratory media were indistinguishable, we observed that CAC1 generated pink colonies on Congo red agar, in contrast to the bright red staining of CO92 colonies grown under the same conditions (data not shown). The MLD for each of the two strains was less than 10 CFU, indicating that the *caf1A*::IS*1541* mutation does not reduce *Y. pestis* virulence in bubonic plague model. As a pneumonic plague model, anesthetized BALB/c mice ($n = 10$) were infected with 1×10^3, 1×10^4, and 4×10^4 CFU of *Y. pestis* CAC1, which revealed that the MLD is 1×10^3 CFU, similar to the MLD of wild-type strain *Y. pestis* CO92 (Fig. 2B). Pneumonic plague-infected animals ($n = 5$) were also killed 24, 48, and 36 h postchallenge to monitor bacterial dissemination from lung tissue to the spleen (Fig. 2C). *Y. pestis* CO92 and CAC1 displayed similar kinetics of replication in lung tissue on day 1 (CO92, 7,700 ± 5,310 CFU; CAC1, 9,400 ± 9,009 CFU; $P \leq 0.726$), day 2 (CO92, $4.05 \times 10^7 \pm 3.72 \times 10^7$ CFU;CAC1, $1.94 \times 10^7 \pm 2.11 \times 10^7$ CFU; $P \leq 0.301$), and day 3 (CO92, $7.6 \times 10^8 \pm 8.0 \times 10^8$ CFU; CAC1, $2.98 \times 10^8 \pm 2.92 \times 10^8$ CFU; $P \leq 0.259$) and similar kinetics of dissemination to the spleen on day 3 (CO92, $4.03 \times 10^8 \pm 5.73 \times 10^8$ CFU; CAC1, $2.7 \times 10^8 \pm 6.04 \times 10^8$ CFU; $P \leq 0.731$) (Fig. 2C). Histopathology analysis of infected lung tissues on the first day of infection revealed very few changes in the lung architecture (Fig. 2D). On the second and third days, alveolar spaces were obstructed with large aggregates of immune cell infiltrates and bacteria (Fig. 2D). Lung parenchyma harbored multifocal areas of necrosis that obliterated the normal lung architecture, indicating that the *caf1A*::IS*1541* insertion does not affect virulence during pneumonic plague (Fig. 2D). These results are similar to the pathology results for pneumonic plague in African green monkeys challenged with F1 antigen-positive (*Y. pestis* CO92) and F1-negative (CO92-C12 and Java-9) strains (13). Davis et al. detected differences between F1-positive and F1-negative strains at necropsy; macroscopic necrohemorrhagic foci were more pronounced in monkeys infected with the F1 mutant strains (13). We could not detect macroscopic differences in the pathological appearance of lung tissues from *Y. pestis* CO92- and *Y. pestis* CAC1-infected mice. All plague-infected animals displayed multilobar pneumonia, and they succumbed to infection at similar rates. Splenomegaly, with variable foci of necrosis, was detected in groups of mice that

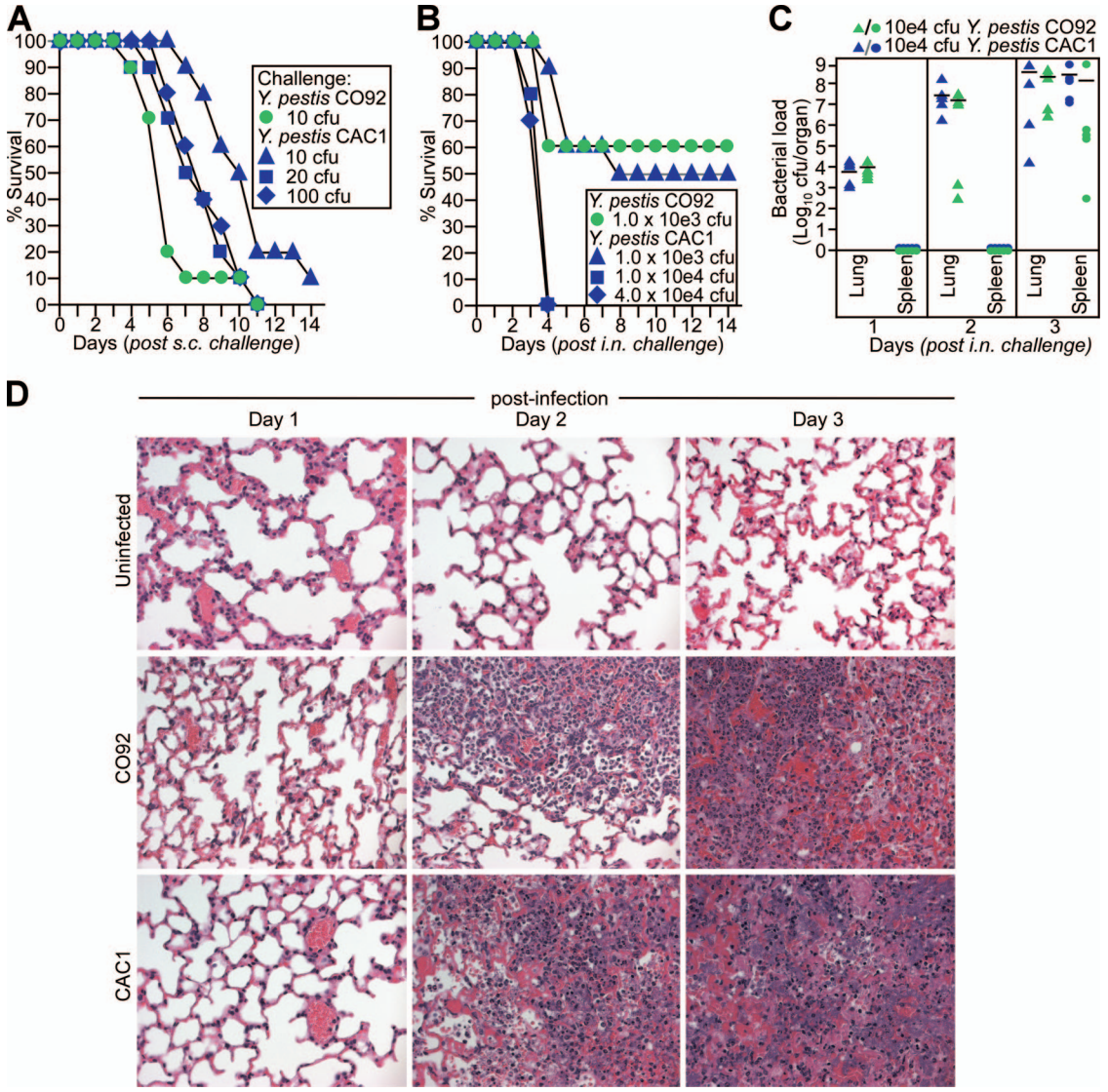

FIG. 2. *Y. pestis* with *caf1A*::IS*1541* is fully virulent. (A) Mice were challenged by subcutaneous (s.c.) injection with the indicated numbers of CFU of *Y. pestis* CO92 (wild type) or CAC1 (*caf1A*::IS*1541*) to precipitate bubonic plague, and their survival was monitored. (B) Strains were inoculated intranasally (i.n.) into mice to precipitate pneumonic plague. (C) *Y. pestis* strains were inoculated intranasally, and lung tissue replication and dissemination of plague bacteria into the spleen were measured on three consecutive days. (D) Lung tissue from animals infected as described above for panel C was fixed, thin sectioned, stained with hematoxylin-eosin, and viewed by microscopy.

had been infected with *Y. pestis* CO92 or CAC1. Histopathology analysis of spleen tissue revealed that the physiological organ architecture had been replaced with bacteria, erythrocytes, edema, cellular debris, and pools of inflammatory cells, predominantly polymorphonuclear leukocytes (Fig. 3), findings that are consistent with those for bubonic plague in cats (61) or humans (25). Similar to the recently reported splenic histopathology observed with ΔF1 mutants (52), we also noted that the severity of splenic tissue destruction appeared to be greater for *Y. pestis* CAC1-infected animals than for *Y. pestis* CO92-infected animals.

***caf1A*::IS*1541* variants escape immunity generated by live attenuated and subunit vaccines.** Due to their ability to generate protection against bubonic and pneumonic plague in humans and animals, live attenuated vaccines have been used extensively in the plague vaccine field (21). Immunization with nonpigmented *Y. pestis* variants generates a large spectrum of antibodies against *Yersinia* surface antigens, including predominantly capsular antigen F1 and, to a much lesser degree, LcrV (48). As a test for vaccine protection against the *caf1A*::IS*1541* mutant, BALB/c mice ($n = 10$) were immunized by intramuscular injection with 1×10^5 CFU *Y. pestis* KIM D27 and challenged 3 weeks later by subcutaneous injection to recapitulate bubonic plague disease. As expected from previous work with a *caf1* deletion mutant of *Y. pestis* CO92 (CO92 ΔF1) (13, 48, 68), immunization with the live attenuated plague vaccine generated protective immunity against challenge with 1,000 MLD of *Y. pestis* CO92 ($P < 0.0001$) and *Y. pestis* CAC1 ($P < 0.001$) compared to mock-immunized (PBS) control animals (Fig. 4A). Following pneumonic plague challenge with 400

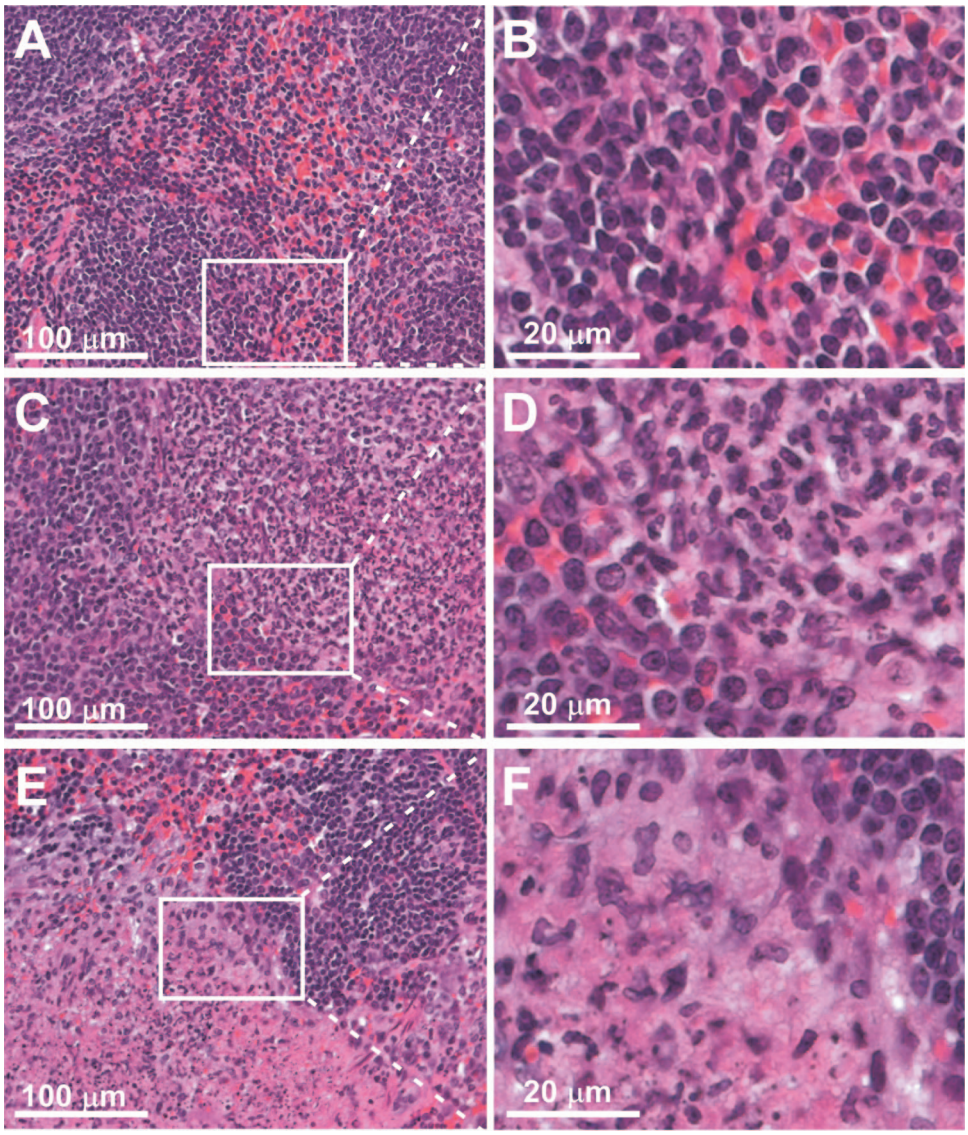

FIG. 3. Spleens from naïve BALB/c mice (A and B) and BALB/c mice infected via subcutaneous injection with 20 CFU of *Y. pestis* CO92 (C and D) or *Y. pestis* CAC1 (E and F) were removed during necropsy on day 7 following the infectious challenge. Tissues were fixed, embedded in paraffin, thin sectioned, stained with hematoxylin-eosin, and viewed by light microscopy at a magnification of ×40. Captured images (A, C, and E) were enlarged fivefold to reveal loss of tissue architecture, necrotic polymorphonuclear leukocytes, massive cellular debris, and edema in plague-infected spleens (D and F) compared to uninfected tissue (B).

MLD of *Y. pestis* CO92, KIM D27-immunized animals displayed full protection ($P < 0.0001$) (Fig. 4B). In contrast, KIM D27-immunized animals succumbed to challenge with 400 MLD of *Y. pestis* CAC1, and the live attenuated vaccine strain did not provide vaccine protection compared with the mock-immunized control ($P = 1$) (Fig. 4B). The pneumonic plague challenge results for the CAC1 mutant strain are similar to those obtained with *Y. pestis* CO92 ΔF1, which also breaks through the protective immunity provided during immunization with KIM D27 (13, 48, 68).

BALB/c animals ($n = 15$) were immunized with purified rF1, rLcrV, rV10 (a variant of LcrV that does not stimulate IL-10 release [15, 43]), as well as combinations of these antigens, all of which generate protective immunity against challenge with >1,000 MLD of *Y. pestis* CO92 in bubonic and pneumonic plague models (48) (data not shown). Subcutaneous bubonic plague challenge with 1,000 MLD of *Y. pestis* CAC1 revealed that there was complete protection with all vaccine formulations that contained rLcrV or rV10, either alone or in combination with rF1 (Fig. 4C). rF1-immunized animals did not display protection against subcutaneous challenge with 1,000 MLD of *Y. pestis* CAC1 ($P = 1$) (Fig. 4C). A similar result was observed following pneumonic plague challenge with 400 MLD of *Y. pestis* CAC1, as all rF1-immunized and mock-immunized control animals succumbed to disease by day 4 (Fig. 4D). Immunization with rV10 or rV10 plus rF1 completely protected mice against challenge with *Y. pestis* CAC1 (rV10 versus PBS, $P < 0.01$; rV10 plus rF1 versus PBS, $P <$

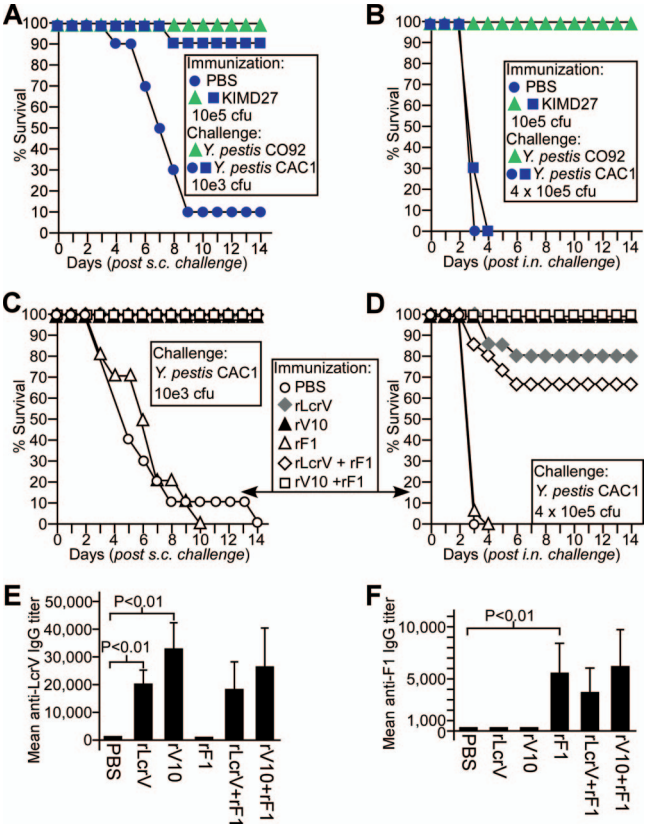

FIG. 4. *Y. pestis* with *caf1A*::IS*1541* escapes plague protective immunity. (A) Mice were immunized with the nonpigmented (Δ*pgm*) strain KIM D27 or mock treated (PBS) and infected by subcutaneous (s.c.) injection with *Y. pestis* CO92 or CAC1 (*caf1A*::IS*1541*), and their survival after bubonic plague challenge was monitored. (B) Mice were immunized as described above for panel A and infected by intranasal (i.n.) instillation, and their survival after pneumonic plague challenge was monitored. (C) Mice were immunized with a mock control (PBS) or with purified subunit vaccines (rLcrV, rV10, rF1, rLcrV plus F1, and rV10 plus F1) and challenged by subcutaneous injection with *Y. pestis* CAC1, and their survival was monitored. (D) Mice immunized as described above for panel C were challenged by intranasal instillation with *Y. pestis* CAC1, and their survival was monitored. Sera from mice immunized with subunit vaccines as described above for panels C and D were examined by ELISA for LcrV-specific (E) and F1-specific (F) IgG antibody titers.

0.01). Immunization of animals with rLcrV and with rLcrV plus rF1 also provided significant protection against *Y. pestis* CAC1 pneumonic plague challenge (rLcrV versus PBS, $P = 0.004$; rLcrV plus rF1 versus PBS, $P = 0.016$); however, 20 and 30% of the immunizied animals succumbed to challenge, respectively (Fig. 4D). Mice immunized with rLcrV or with rLcrV plus rF1 displayed lower titers of LcrV-specific antibodies than animals immunized with rV10 displayed (Fig. 4E and F).

***caf1A*::IS*1541* variants escape the opsonophagocytic attributes of F1 antibodies.** Mouse antibodies directed against F1 promote phagocytic clearance of *Y. pestis* in human blood otherwise lacking plague-specific antibodies and overcome the virulence attributes of the pathogen's type III secretion machinery with LcrV (17) (Fig. 5A). In the absence of antibodies against LcrV or F1, *Y. pestis* employs the type III machinery to transport effector Yops into immune cells (49), which rapidly

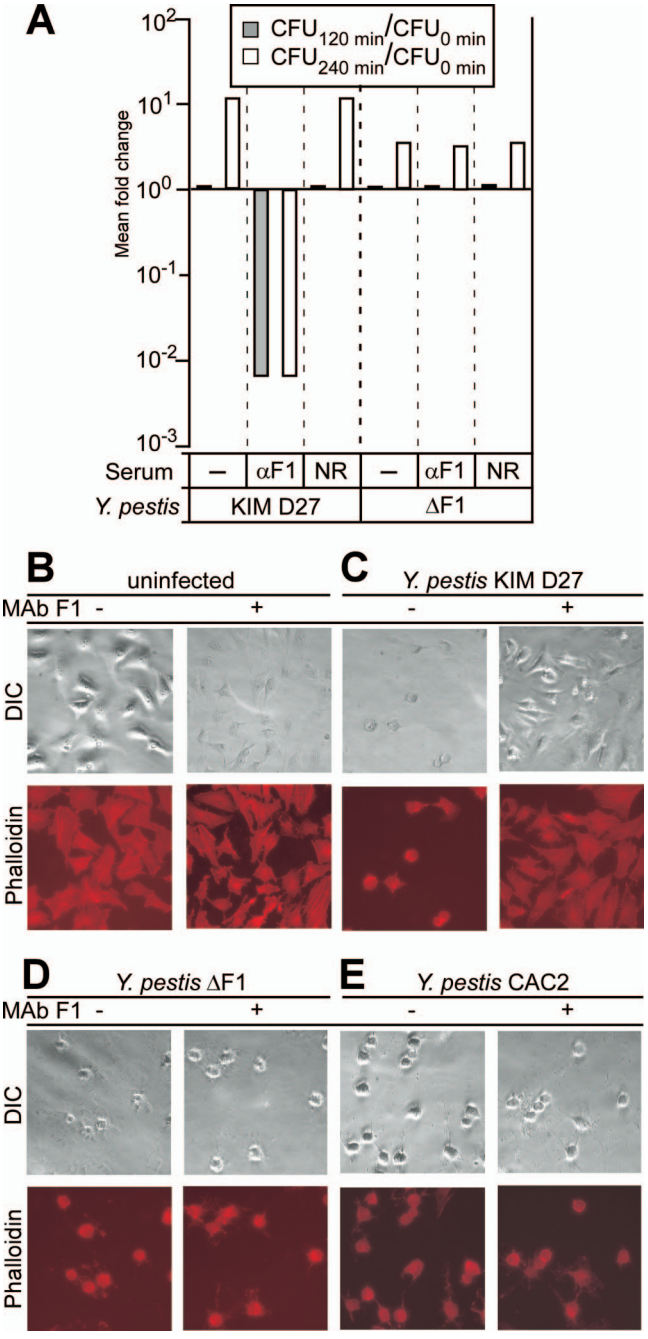

FIG. 5. F1 antibodies block *Y. pestis* type III injection, which is overcome by *caf1A*::IS*1541*. (A) Human anticoagulated blood was incubated for 120 or 240 min with 10^5 CFU *Y. pestis* KIM D27 or the isogenic ΔF1 mutant strain in the absence (−) or presence of mouse anti-F1 (αF1) or a nonreactive serum control (NR). Mean changes in bacterial load were recorded by plating aliquots on agar and incubating the preparations for colony formation (CFU). (B to E) HeLa tissue culture cells were viewed by differential interference contrast (DIC) microscopy, or their actin cables were stained with rhodamine-phalloidin and viewed by fluorescence microscopy. Tissue culture cells were either not infected (B) or were infected with plague strains in the presence or absence of 500 μg F1-specific MAb F1-04-A-G1 (MAb F1) using *Y. pestis* KIM D27 (C), ΔF1 (*caf1* deletion) (D), and CAC2 (*caf1A*::IS*1541*) (E).

depletes host supplies of phagocytes and associated innate immune defenses (37). Antibodies against LcrV block type III injection and provide protective immunity (14, 47, 62). To test whether the *caf1A*::IS*1541* mutation enables *Y. pestis* to escape the presumed effect of F1 antibodies to interfere with type III injection of effector Yops, we monitored *Yersinia* type III injection of HeLa tissue culture cells and measured the pathological attributes of effector Yops on actin arrangements with fluorescence microscopy and rhodamine-labeled phalloidin (49) (Fig. 5B). Mouse antibodies raised against rF1 interfered with the ability of *Y. pestis* to catalyze type III injection (data not shown). Similar to mouse polyclonal anti-F1, MAb F1-04-A-G1 provides protective immunity against plague when it is passively transferred into mice (1) (data not shown). Addition of MAb F1-04-A-G1 to HeLa cells blocked *Yersinia* cytotoxicity in HeLa cells (Fig. 5C). Deletion of the structural gene for F1 pili (*caf1*) from *Y. pestis* KIM D27 enabled isogenic ΔF1 mutants to escape the type III inhibitory attributes of F1-specific MAbs (Fig. 5D). The *caf1A*::IS*1541* mutation had the same effect and restored *Y. pestis* type III injection even in the presence of F1-specific antibodies (Fig. 5E). As controls, neither *caf1A*::IS*1541* nor deletion of *caf1* affected *Y. pestis* type III secretion in vitro or type III-mediated cytotoxicity in HeLa cells (data not shown). Further, mock treatment of *Yersinia*-infected HeLa cells with 500 μg murine anti-C-myc MAb (Fitch Monoclonal Antibody Facility, University of Chicago) had no effect on cytotoxicity following *Y. pestis* KIM D27 or CAC2 infection of HeLa cells (data not shown).

DISCUSSION

Type III injection of effector Yops is an essential virulence mechanism of *Y. pestis* (46), and LcrV plays an essential role by gating this transport pathway at the tip of its needle structures (41). Immunization with purified LcrV antigen generates specific antibodies that block type III injection (47) and confers protective immunity against plague (7). However, these antibodies are not sufficiently developed in plague-infected animals or in animals immunized with live attenuated vaccine strains (10, 48). Instead, humoral immune responses are directed largely at a capsular antigen (F1 pili) (10, 48). F1-specific antibodies enable phagocytic clearance (9) and, as suggested here, likely impact *Yersinia* type III injection (17). These results aid in the appreciation of protective immunity against plague, as research into the discovery of additional plague protective antigens has thus far failed to identify envelope components that provide levels of vaccine protection comparable to that described for LcrV and F1 (12, 55). Although a molecular mechanism whereby F1 antibodies may impact *Y. pestis* type III injection into host cells is not known yet, we think that it is conceivable that the abundant deposition of antibodies at the dense layer of F1 pili could prevent interaction of needle complexes harboring LcrV with their target cells.

We report that IS*1541* insertion into *caf1A* enables *Y. pestis* to escape the protective immunity of F1 antibodies derived from either purified antigen subunits or live attenuated vaccine strains. IS*1541* resembles the IS*200* element of *Salmonella enterica* serovar Typhimurium (85% identity); six copies of IS*200* are present in strain LT2, and this element is mobilized infrequently but gives rise to insertion mutants and recombinational chromosome rearrangements (22, 31, 32). *Y. pestis* CO92 carries 66 complete or partial copies of IS*1541* on its chromosome or virulence plasmids (44), 1 of which has been found in the structural gene for invasin (*inv*) (54), an outer membrane ligand for β1-integrins that plays an important role in gastrointestinal infection by *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (26, 27) but otherwise is dispensable for plague infection (50). The remaining copies of IS*1541* are located outside large open reading frames (44). Although the number and genome location of IS*1541* elements vary in currently known plague isolates, which contain 43 to 62 chromosomal copies, mobilization of this element has not been reported previously (42, 60). Our preliminary attempts to quantify IS*1541* insertion into the *caf1A* target sequence when *Y. pestis* KIM D27 is grown on HIA have not been successful, suggesting that such events may be rare under laboratory growth conditions (data not shown). Nevertheless, in vivo IS*1541* insertion occurs frequently enough to permit isolation of the CAC1 variant from a mouse that was challenged with 10^7 CFU of *Y. pestis* CO92.

The *Y. pestis caf1A*::IS*1541* variant is fully virulent in bubonic and pneumonic models of disease. It is conceivable that the IS*1541* insertion element may allow plague bacteria to replicate in its variable hosts for longer periods of time, because the *caf1A*::IS*1541* insertion would enable mutants to escape adaptive immune responses and disseminate to new hosts. As *caf1A*::IS*1541* variants escape plague immunity derived from rF1 subunits and whole-cell live attenuated vaccine strains, in the future efforts to develop human plague vaccines should consider *caf1A*::IS*1541* mutants in the spectrum of plague strains for which protection must be achieved. Due to the inclusion of a second protective antigen, it seems likely that subunit vaccines containing purified F1 and LcrV (24, 66) can generate humoral immune responses that protect even against *caf1A*::IS*1541* mutants.

F1 mutant strains have been isolated previously from mice (8) and from rats that developed fatal plague infections but had received prior immunization with the live attenuated plague vaccine (EV76) (64, 65). Previous work revealed an F1-negative strain with a spontaneous 66-bp deletion in the *caf* promoter that affects expression of the *caf* operon (18). Perhaps the most comprehensive analysis of naturally occurring plague variants with altered expression of *Y. pestis* major virulence determinants was conducted by the Anti-Plague Establishment of the former Soviet Union (3). Approximately 7% of all isolates are affected in expression of *lcr*, *pgm*, *pla*, *caf1*, *ymt*, or *psa*, and 15% of these isolates lack *caf1* (F1) expression (3). Future work will need to examine the molecular mechanisms and frequency with which F1 variants can be generated.

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