INFECTION AND IMMUNITY, Aug. 2006, p. 4809–4816 Vol. 74, No. 8
0019-9567/06/$08.00+0 doi:10.1128/IAI.00034-06

Virulence of *Francisella* spp. in Chicken Embryos

Eli B. Nix,[1] Karen K. M. Cheung,[1] Diana Wang,[2] Na Zhang,[1] Robert D. Burke,[1,2] and Francis E. Nano[1]*

Department of Biochemistry and Microbiology[1] and Department of Biology,[2] University of Victoria, Victoria, British Columbia, Canada

Received 6 January 2006/Returned for modification 7 February 2006/Accepted 9 May 2006

We examined the utility of infecting chicken embryos as a means of evaluating the virulence of different *Francisella* sp. strains and mutants. Infection of 7-day-old chicken embryos with a low dose of *F. novicida* or *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) resulted in sustained growth for 6 days. Different doses of these two organisms were used to inoculate chicken embryos to determine the time to death. These experiments showed that wild-type *F. novicida* was at least 10,000-fold more virulent than the LVS strain. We also examined the virulence of several attenuated mutants of *F. novicida*, and they were found to have a wide range of virulence in chicken embryos. Fluorescent microscopic examination of infected chicken embryo organs revealed that *F. tularensis* grew in scattered foci of infections, and in all cases the *F. tularensis* appeared to be growing intracellularly. These results demonstrate that infection of 7-day-old chicken embryos can be used to evaluate the virulence of attenuated *F. tularensis* strains.

Francisella tularensis is a highly infectious, gram-negative bacterial pathogen with a 50% infectious dose of less than 10 cells for most animals (17). Although all strains are highly infectious, there is great variety in the morbidity and mortality that each strain is able to induce in different host animals. *F. tularensis* subsp. *tularensis* biovar "type A" is clearly the most virulent biovar in humans and laboratory animals. The type A biovar is found naturally only in North America, where it caused high mortality rates before the availability of antibiotics (18). In one survey 19% of tularemia patients died (12), but it is not known what proportion of these patients were infected with a biovar type A strain. Even with antibiotic treatment infection of humans with this subspecies results in some deaths. Recently, the type A biovar has been separated into two clades (24), but the relative levels of virulence of the two groups have not been studied. *F. tularensis* subsp. *holarctica* is found throughout the Northern Hemisphere. Although it is highly infectious in all of the animals that it infects and is fatal to mice (7), this subspecies rarely causes death in humans, although it can cause considerable morbidity. The live vaccine strain (LVS) of *F. tularensis* subsp. *holarctica* and *F. novicida* have been widely used as models of *F. tularensis* infection, primarily because these bacteria have low virulence in humans and can be handled in BSL2 facilities (3, 14, 16, 19). In the mouse model of infection the LVS has an intradermal 50% lethal dose (LD_{50}) of about 3×10^5 CFU, and *F. novicida* has an LD_{50} of about 2×10^3 CFU (25); however, both strains have an intraperitoneal LD_{50} of less than 10 organisms. Hence, mouse infections with LVS and *F. novicida* may be approximations of *F. tularensis* type B and type A infections in humans, respectively.

F. tularensis is thought to grow primarily inside cells during infection of animals. In vitro studies of intramacrophage growth have shown that initially *F. tularensis* resides in a phagosome, from which it largely escapes between 2 and 4 h after cell entry (8, 20). The *F. tularensis*-laden phagosome has a relatively neutral pH and accumulates some markers of late endosomes, such as LAMP1 and CD63, while it excludes another late endosome marker, cathepsin D (8). Expression of the *F. tularensis* protein IglC is required for escape of *F. tularensis* from the phagosome, but its role is unknown (29). The live vaccine strain of *F. tularensis* has been shown to induce apoptosis in the J774 mouse macrophage cell line (26) and to inhibit secretion of tumor necrosis factor alpha and interleukin-1 (38). Although the suppression of cytokines probably represents an *F. tularensis* virulence strategy, the induction of apoptosis likely reflects a defensive response of the host, as caspase-1 knockout mice are more susceptible to *F. tularensis* infection (30).

A small number of virulence factors have been identified in *F. tularensis*, and most of these factors affect intramacrophage

TABLE 1. *Francisella* sp. strains

Strain	Genotype or phenotype	Reference
LVS	Live vaccine strain of *F. tularensis* subsp. *holarctica*	13
U112	Wild-type *F. novicida*	27
SC66	U112 derivative; lacks complete O-antigen; delayed growth in thioglycolate-induced C57BL/6 peritoneal macrophages	10
CG57	U112 derivative with insertion in *purA* gene affecting purine biosynthesis; suppressed growth in thioglycolate-induced C57BL/6 peritoneal macrophages	22
CG69	U112 derivative with insertion in *clpB* gene which encodes a heat shock response protease; suppressed growth in thioglycolate-induced C57BL/6 peritoneal macrophages	22
NZ9	U112 derivative in which the *pdpA* gene is replaced with an erythromycin resistance cassette; unable to grow in macrophages	33
GB2	U112 derivative; point mutation in *mglA* which encodes a global regulator of virulence; unable to grow in macrophages and avirulent in mice	5

* Corresponding author. Mailing address: Department of Biochemistry and Microbiology, PO Box 3055 STN CSC, University of Victoria, Victoria, B.C. V8W 3P6, Canada. Phone: (250) 721-7074. Fax: (250) 721-8855. E-mail: fnano@uvic.ca.

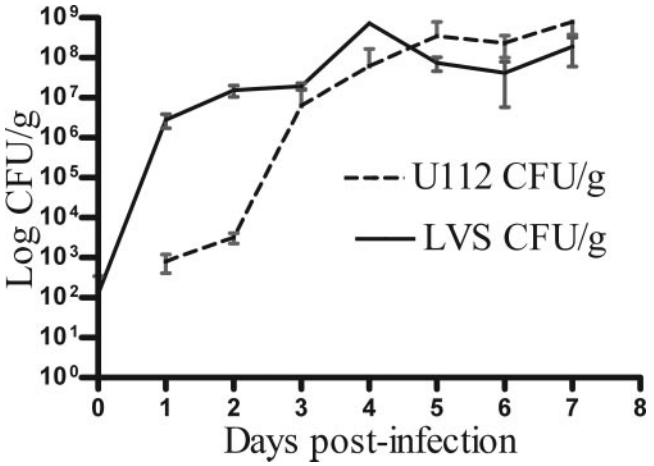

FIG. 1. Growth of *F. tularensis* LVS and *F. novicida* in chicken embryos. Twenty-one chicken embryos were infected with 168 CFU of LVS or 159 CFU of *F. novicida* U112. Immediately after infection and every 24 h after infection the bacterial burden of three embryos was determined. At zero time for the U112 strain there were too few bacteria to count.

growth. Inactivation of the *mglAB* global regulatory genes results in strains whose growth is severely hampered in macrophages (5). Presumably, MglA and MglB are required for transcription of genes encoding effector proteins, especially genes found in the *Francisella* pathogenicity island (28). There is genetic evidence that the *Francisella* pathogenicity island-associated genes *iglA*, *iglC*, *pdpA*, and *pdpD* are required for intramacrophage growth (21, 22, 33). There is biochemical (23) and genetic (36) evidence that a capsule exists and is needed for infectivity and virulence. Defects in the production of lipopolysaccharide can affect intracellular growth (10, 31). The observed in vitro intracellular growth and the requirement for cell-mediated immunity for clearance (1, 4, 9, 14) of an *F. tularensis* infection suggest that intracellular growth is required for virulence in animals. The observation that mutants defective for growth in macrophages are also less virulent in animals supports this notion (31, 33).

F. tularensis infects a wide variety of animals, and several animals, including rabbits, guinea pigs, primates, hamsters, rats, and mice, have been used as models of infection (2, 14–16, 32, 34, 37). Chicken embryos have also been used to test *F. tularensis* virulence and pathology (6, 35). Recently, researchers have begun to use simple biological systems, such as the nematode *Caenorhabditis elegans* (11), flies, and insect larvae

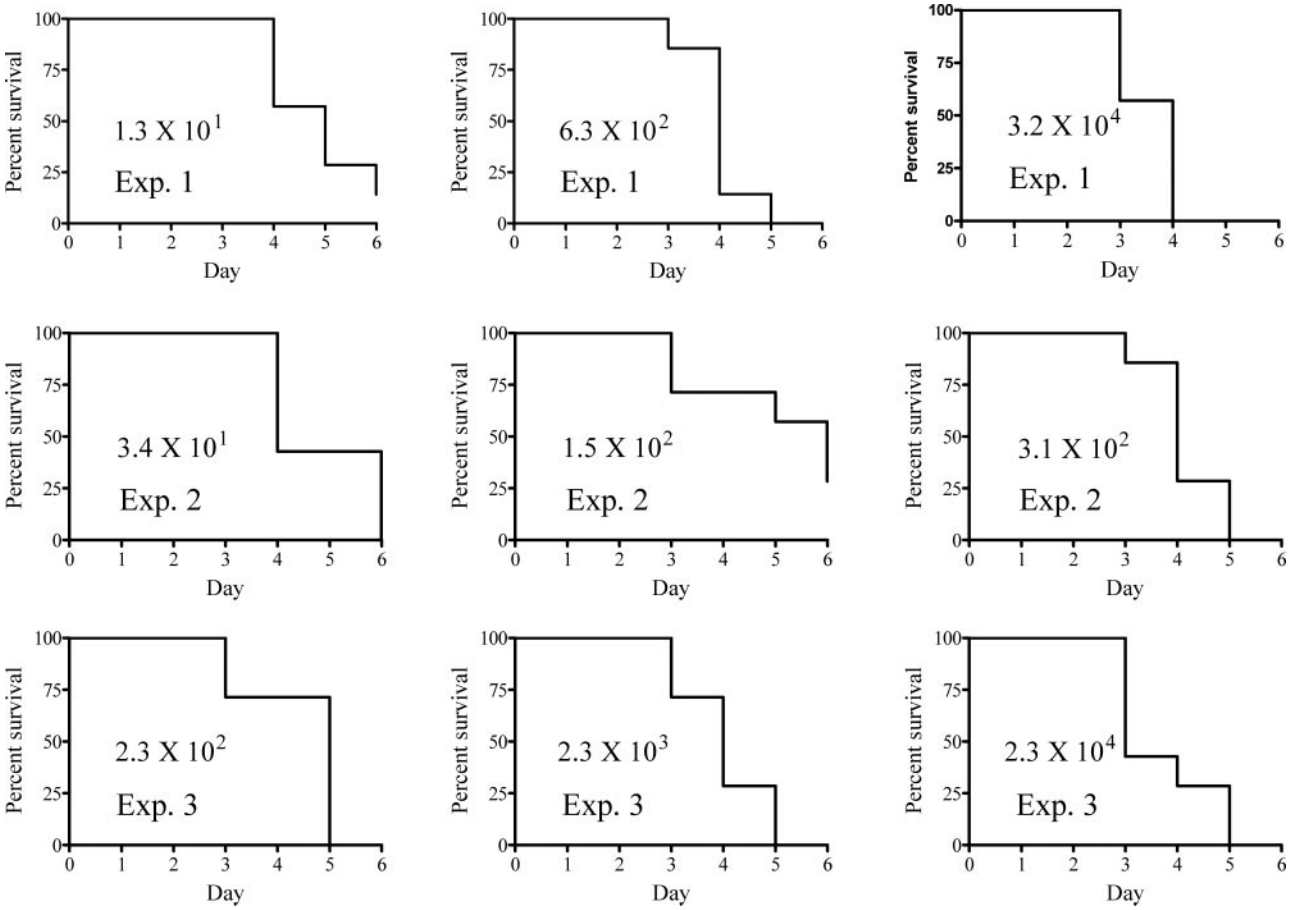

FIG. 2. Reproducibility of the time to death induced by *F. novicida* U112. Three inocula that were different sizes were each used for infection of seven fertilized eggs. The inoculum used for infection (in CFU) is indicated on each graph. In each experiment eggs were infected on separate days and different lots of fertilized eggs were used.

(39), to examine the virulence properties of bacterial pathogens. Such systems permit large-scale testing that is humane and relatively inexpensive. Our objective in this work was to develop an assay system that allowed us to evaluate the virulence of *F. tularensis* strains without having to infect animals that have fully developed nervous systems.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *F. tularensis* strains used in this study are listed in Table 1. All of the *F. novicida* strains were derived from the prototype strain U112 (= ATCC 15482), which had been passaged through a mouse and aliquoted for subsequent experiments. The LVS (ATCC 29684) was obtained from the American Type Culture Collection. Strains were grown aerobically at 37°C in either tryptic soy broth or on tryptic soy agar (TSA) supplemented with 0.1% L-cysteine.

Chicken embryo infections. *F. tularensis* strains were grown to the late log phase (optical density at 600 nm, 0.9 to 1.0) and diluted in phosphate-buffered saline (PBS) (Gibco) for injection. The inoculating dose was calculated retrospectively by determining the CFU following dilution and plating on TSA. Fertilized White Leghorn eggs were obtained from the University of Alberta Poultry Research Station. Chicken embryos were incubated at 37°C with high humidity for 7 days prior to infection and throughout the experiment were mechanically tilted to a 45° angle every 40 min. After the initial 7-day incubation the eggs were examined in order to discard those that lacked a viable embryo, a phenomenon that occurred in between 5 and 10% of the fertilized eggs. For inoculation the tops of the egg shells were disinfected with 70% ethanol. A 1-cm-diameter window was made in the air sac end of an egg, and the egg shell membrane was reflected. With a tuberculin syringe, 100 μl of inoculum was injected under the chorioallantoic membrane. After injection the shells were sealed with clear packing tape. Eggs were candled to detect signs of death every 24 h for 6 days. This process consisted of shining a focused bright light at one end of an egg to determine if the network of blood capillaries was intact. Embryos that died within 24 h of inoculation were assumed to have suffered lethal trauma during the inoculation and were removed from the experiment. For the study of the time course of *F. tularensis* growth, the chicken embryos were killed by incubating the eggs in a −20°C freezer for 1 h, followed by blending of the eggs and plating dilutions of the homogenized egg contents on TSA to determine the bacterial load. All experiments were terminated by the time the embryos were 14 days old. Chicken embryos that were less than 17 days old are not subject to regulatory control in many countries, including Canada.

Microscopy. Embryos were removed from shells and rinsed with PBS, and organs of interest were removed by dissection and fixed overnight with PBS containing 4% paraformaldehyde. The organs were rinsed with PBS, infiltrated with OCT compound (Tissue-Tek 4583), and snap frozen with liquid nitrogen, and then they were transferred to −80°C for storage. Ten-micrometer cryostatic sections were cut and mounted on gelatin-coated slides. The tissue sections were blocked with 5% lamb serum–PBS containing 0.05% Tween 20 at room temperature for 45 min. Primary antibodies were diluted 1:1,000 in 5% lamb serum–PBS and incubated overnight at 4°C with the sections. After several washes in PBS, sections were incubated with Alexa 488 or 568-conjugated (Molecular Probes) goat anti-rabbit or goat anti-mouse secondary antibodies for 2 h at room temperature, rinsed with PBS, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) After mounting with a coverslip, specimens were examined with a Leica DM-6000 compound microscope.

Digital images were collected, and color was added with OpenLab (version 4.04) or Scion Image (version 4.03). Figures were prepared with Photoshop (Adobe 6.0) by cropping and adjusting the brightness and contrast.

RESULTS AND DISCUSSION

Growth of *F. tularensis* in 7-day-old chicken embryos. When a small inoculum of *F. tularensis* subsp. *holarctica* LVS or *F. novicida* was introduced into chicken embryos, exponential growth occurred for 4 to 5 days, and the concentration reached about 5×10^8 bacteria per g of egg mass (Fig. 1). *F. novicida* and LVS appeared to grow at approximately the same rate, and the concentrations leveled off at similar total numbers of bacteria by day 5 postinfection.

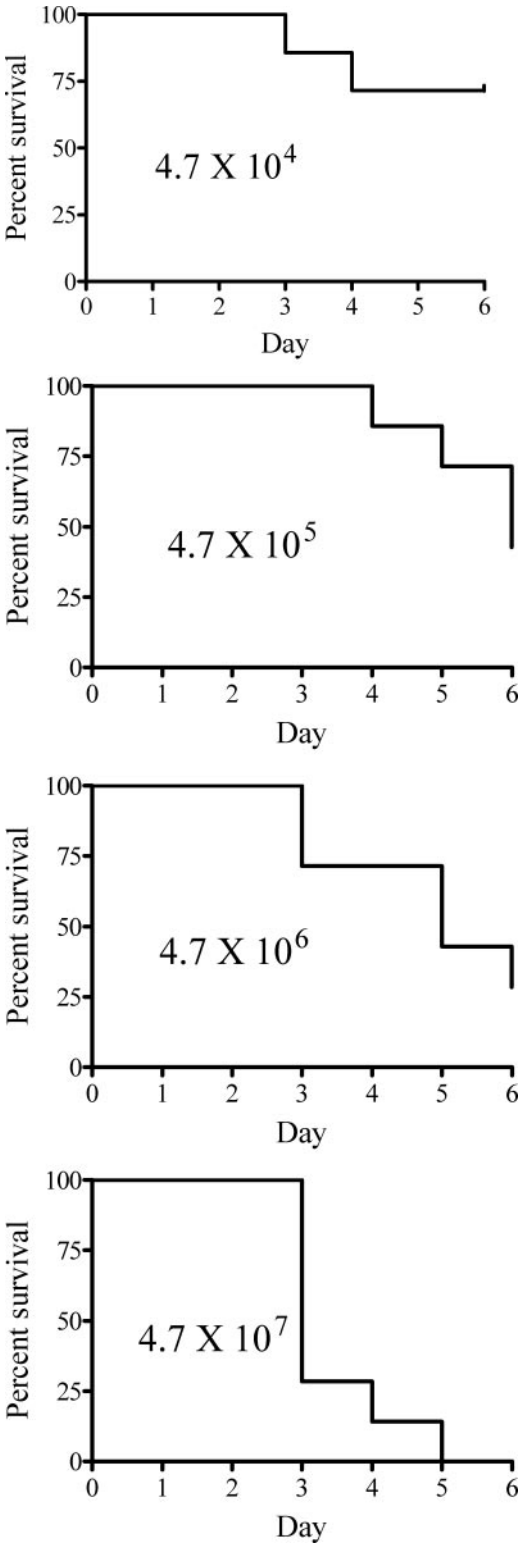

FIG. 3. Virulence of *F. tularensis* LVS in chicken embryos. Seven fertilized eggs were infected using each inoculation dose, and the doses (in CFU) are indicated on the graphs. The infection series was repeated two times, and similar results were obtained.

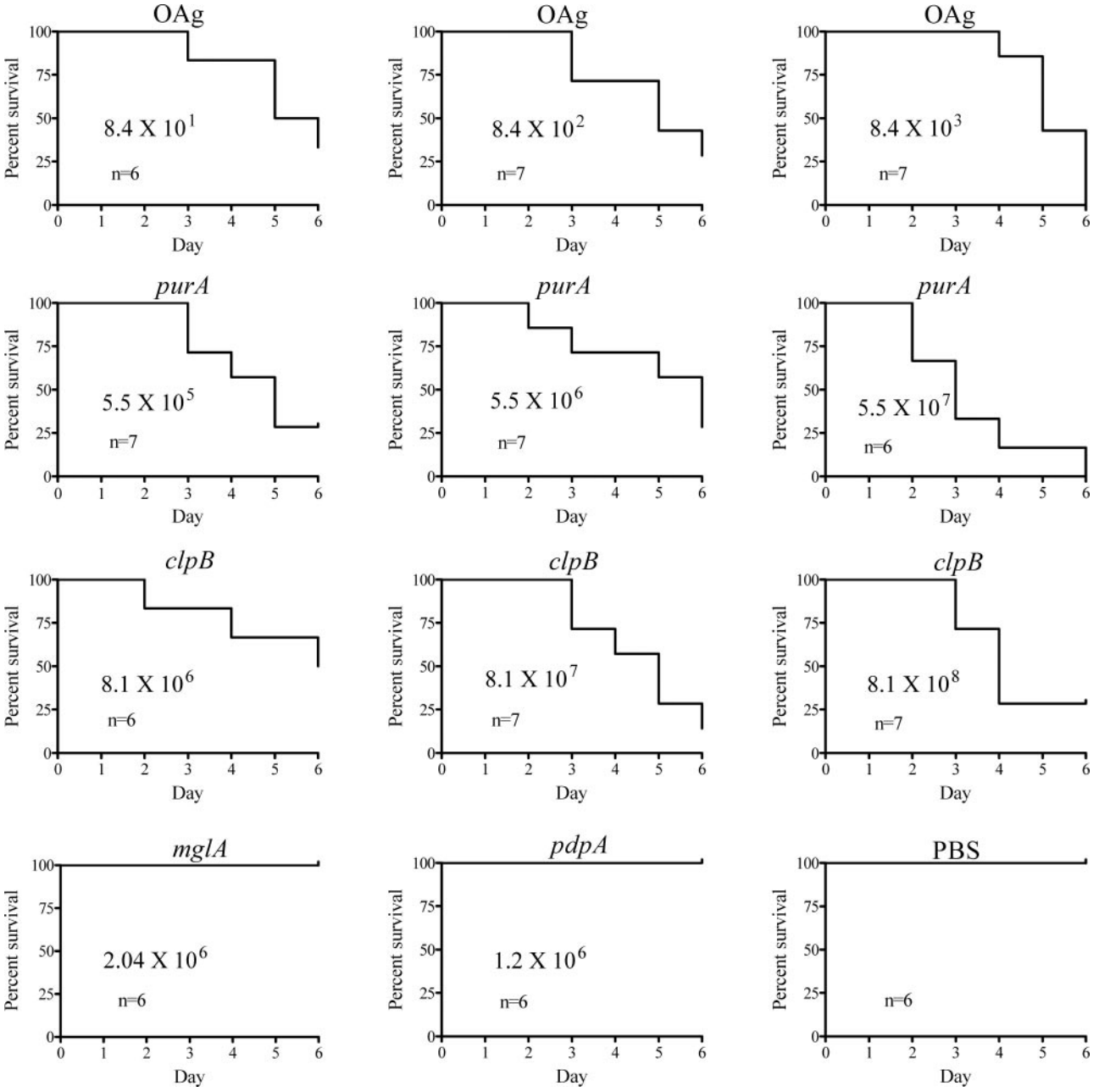

FIG. 4. Levels of virulence of *F. tularensis* strains in chicken embryos. A dilution series was prepared for each *F. tularensis* strain, and seven embryos were infected with each inoculating dose. The death of the embryos was monitored each day. In some instances the embryos died within 24 h of inoculation (indicated by $n = 6$), and such deaths were attributed to injury induced during the inoculation and thus were discounted. The number in each graph indicates the inoculating dose (in CFU). The graphs labeled OAg, *purA*, *clpB*, *mglA*, and *pdpA* indicate the results for infections with strains SC66, CG57, CG69, GB2, and NZ9, respectively. The virulence of each strain was tested at least twice, and similar results were obtained in all trials.

Virulence of *F. novicida*. The wild-type strain of *F. novicida* is highly virulent in mice and also appears to be highly virulent in chicken embryos, and 100% lethality was observed with 30 to 200 CFU (Fig. 2). Infections with different doses and repetitions of experiments with different lots of eggs demonstrated that the time to death due to infection generally correlated with the infectious dose and was consistent for different experiments. Some aberrations were seen, like the results obtained with the intermediate dose given in experiment 2, and these aberrations may have been due to the natural variation in embryos or to the inexact nature of the inoculation. All of the embryos inoculated with phosphate-buffered saline lived until the termination of the experiment on day 6 postinfection (data not shown).

Virulence of *F. tularensis* LVS. In the mouse *F. novicida* is about 100-fold more virulent than LVS when both organisms

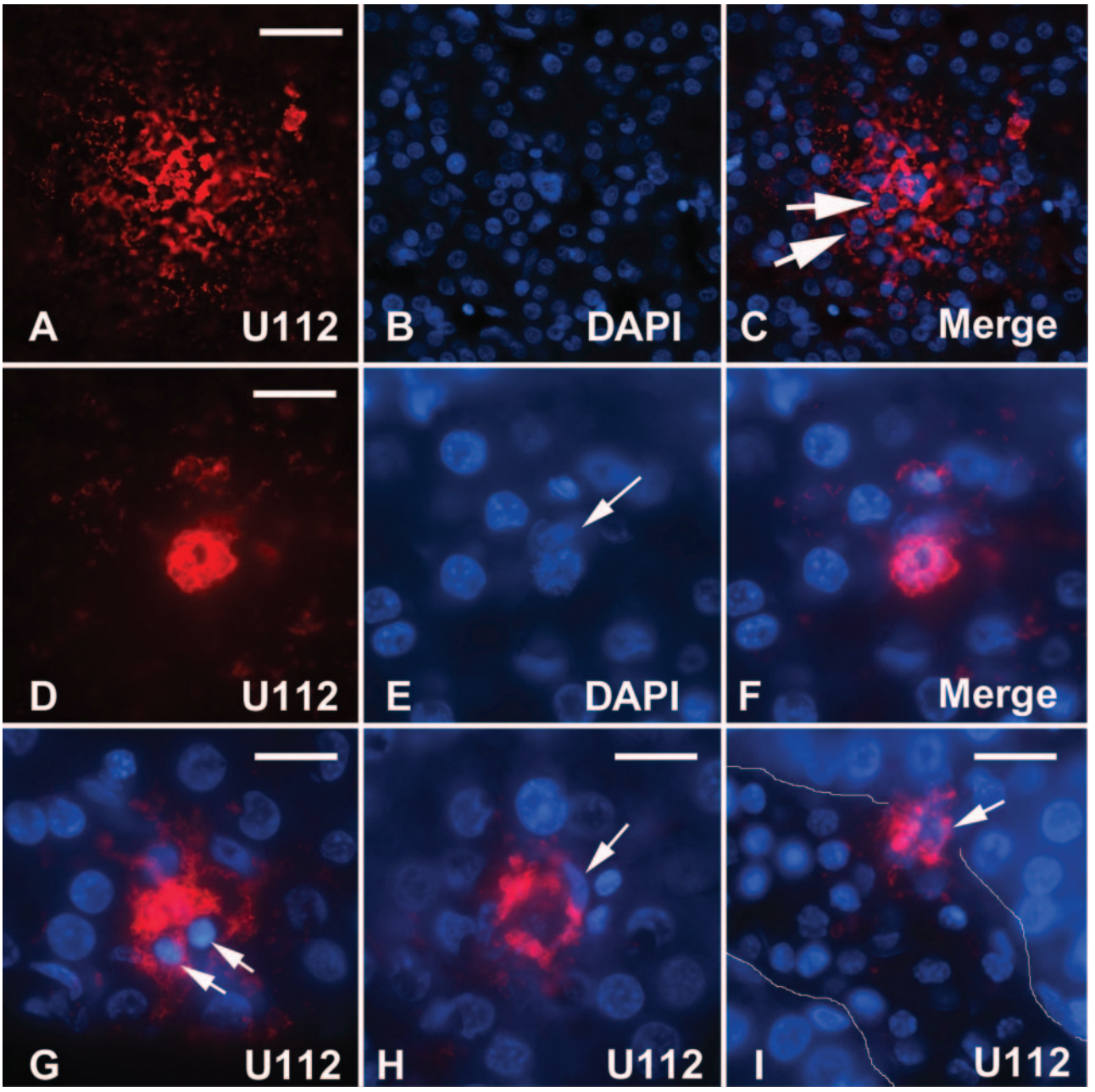

FIG. 5. Immunofluorescence of *F. novicida* U112 in chicken embryonic tissues. (A to C) Focal infections in livers resulted in intense immunoreactivity in the cytoplasm of the central cells and a gradation of immunoreactive material in surrounding cells. The arrows indicate individual nuclei that were surrounded by immunoreactive cytoplasm. (D to F) Higher magnification, showing that the immunoreactive material in the cytoplasm also stained with DAPI in a granular pattern consistent with intracellular accumulation of bacterial cells (arrow). (G) In some liver cells the nuclei of infected cells were pyknotic and fragmented (arrows), which was indicative of an apoptotic response. (H) In some instances cells associated with sites of infection had the flattened nuclear morphology of liver macrophage cells. (I) Infected cells (arrow) were also associated with the walls of blood vessels (indicated by a thin white line). Nucleated erythrocytes are present in the blood vessels. All images are images of tissue from embryos at 3 days postinfection, and all tissues were reacted with anti-*F. novicida* rabbit serum. (A to C) Bar = 25 μm. (D to I) Bars = 15 μm.

are delivered via intradermal injection. Infection of chicken embryos revealed an even larger difference in virulence between the two organisms, and *F. novicida* was at least 10,000-fold more lethal than *F. tularensis* LVS (Fig. 2 and 3).

Virulence of mutants of *F. novicida*. The virulence of the strains listed in Table 1 was evaluated by performing a series of infection experiments using different numbers of bacteria as inocula and determining the time to death of the chicken embryos. First, a pilot experiment was performed for each mutant to determine the approximate lethal dose, and then at least two repetitions of experiments were carried out using inoculum concentrations in the sublethal to lethal range. For

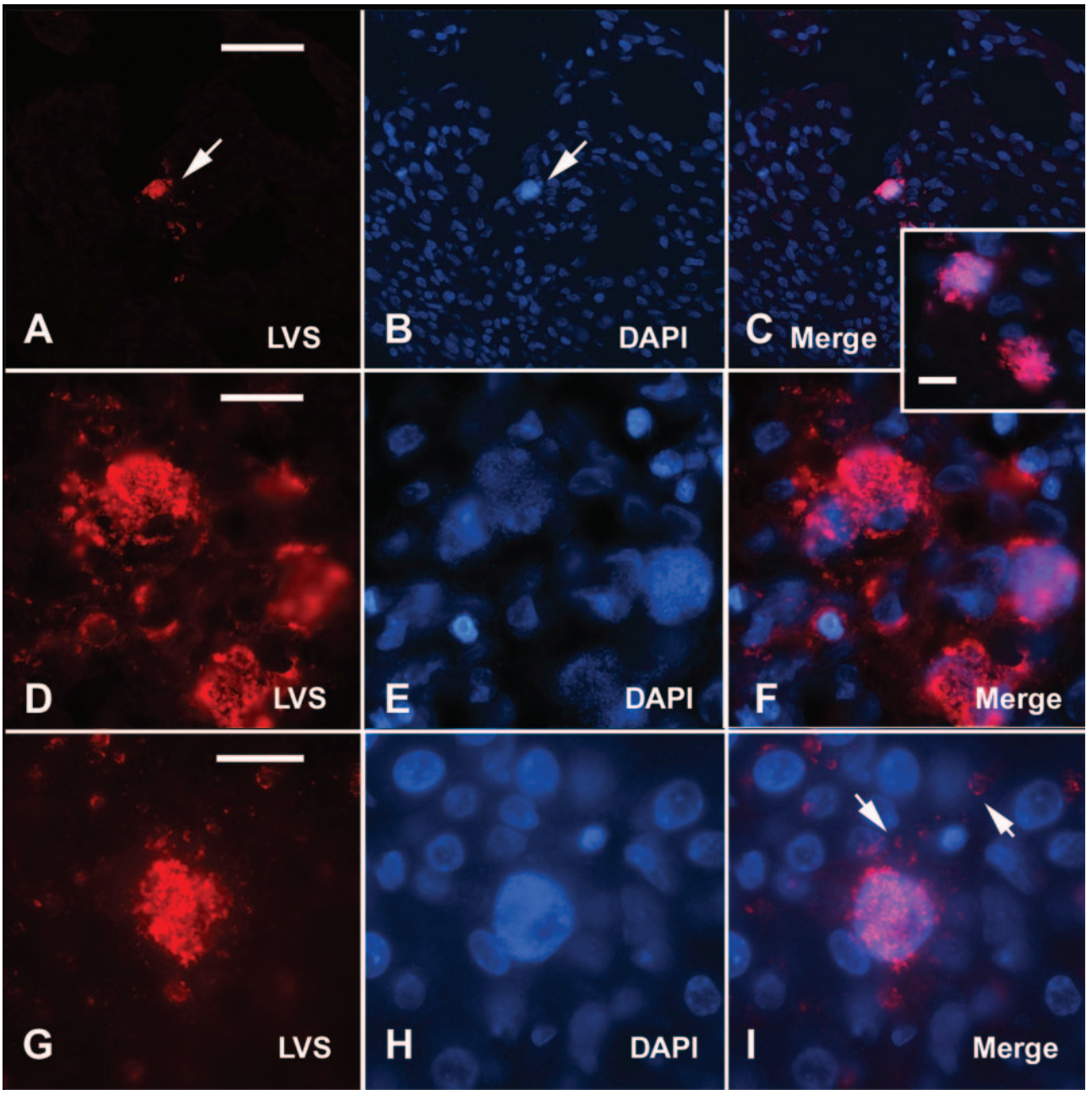

FIG. 6. Immunofluorescent localization of *F. tularensis* LVS in chicken embryonic tissues. (A to C) In embryos 2 days after inoculation, immunoreactive cells were scattered throughout embryonic tissues. In sections through the heart immunoreactivity and characteristic granular DAPI staining were tightly clustered around individual heart cell nuclei (arrows and inset). (D to F) In embryos 4 days after inoculation, large foci of immunoreactive material were present in most tissues. Details of infected cells suggested that bacterial cells were in individual heart cells. (G to I) Focal infections also occurred in the liver 6 days after infection, and in individual cells there was granular immunoreactive material in the cytoplasm that also stained with DAPI. There was scattered immunoreactive material in the surrounding cells (arrows). Panels A to F show tissues that were reacted with anti-*F. tularensis* LVS monoclonal antibody that is reactive with O-antigen. Panels G to I show tissues that were reactive with anti-*F. tularensis* LVS rabbit serum. (A to C) Bar = 25 μm. (D to I) Bars = 15 μm. (Inset) Bar = 5 μm.

each inoculating dose seven eggs were infected. Mutants of *F. novicida* U112 which had previously been shown to have defects in virulence or growth in macrophages displayed a range of virulence levels in chicken embryos. In total, the mutant strains were about 100-fold less virulent to more than 100,000-fold less virulent (Fig. 4) than the wild-type *F. novicida* U112 strain. Mutant GB2, which has a defect in the global regulator MglA, and mutant NZ9, which has a substitution in the pathogenicity island gene *pdpA*, are both avirulent in mice and were found to be unable to kill any of the six chicken embryos that were infected with inocula larger than 10^6 CFU. The previously described mutants SC66, CG57, and CG69 exhibited intermediate levels of virulence. Strain SC66, which has a defect in O-antigen production, required an inoculum that was about

100 times that of its parent strain, strain U112, to induce 100% lethality. A *purA* mutant, CG57, was shown to be attenuated about 10,000-fold compared to wild-type *F. novicida*. Finally, mutant CG69, which has an insert in the gene encoding the heat shock-induced protease ClpB, was shown to be attenuated about 10^6-fold compared to the wild-type strain.

***F. tularensis* in chicken embryonic tissue.** Infected chicken embryos were surveyed by microscopy to localize tissues that support *F. tularensis* growth. Embryos that had been infected with the U112 strain or LVS were sectioned, and bacteria were visualized with strain-specific antisera or monoclonal antibodies (Fig. 5 and 6). Two days after infection of the embryos, the immunoreactive foci were small and widely dispersed (Fig. 6A to C). Immunoreactive material was found in all of the tissues examined, including the heart, liver, kidney, and bone marrow. For both the U112 strain and LVS immunoreactivity was found inside individual chicken cells in which distinctive granular DAPI staining dominated the cytoplasm. Four days after infection, immunoreactive foci were abundant and were present throughout embryonic tissues. Bacterial cells appeared to be contained within chicken cells, and the infected cells were typically in clusters (Fig. 5A to F and 6D to I). Often, lesions had necrotic tissue at the center that was surrounded by a ring of infected cells. In some instances the host cell nucleus was pyknotic, which is typical of an apoptotic response (Fig. 5G).

These studies indicate that the chicken embryo system is a useful system for evaluating the virulence of *F. tularensis* strains. *F. tularensis* grew vigorously in chicken embryos and induced death within 1 week after inoculation. The bacteria were intracellular, and the structure of the lesions suggested that the initial infections spread by localized infection of adjacent cells. The use of chicken embryo infection is not intended to replace mammalian models of tularemia, which involve complex immune responses and approximate human tularemia. However, despite its flaws, *F. tularensis* infection of chicken embryos provides a rapid, inexpensive test to determine differences in levels of bacterial strain virulence that does not induce pain in animals. This procedure could be combined with in vitro intracellular assays to screen mutants, allowing researchers to plan subsequent animal experiments so that the minimal number of animals is used.

ACKNOWLEDGMENT

This work was supported by grant 5R01 AI056215-02 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Allen, W. P.** 1962. Immunity against tularemia: passive protection of mice by transfer of immune tissues. J. Exp. Med. **115:**411–420.
2. **Anthony, L. S. D., R. D. Burke, and F. E. Nano.** 1991. Growth of *Francisella* spp. in rodent macrophages. Infect. Immun. **59:**3291–3296.
3. **Anthony, L. S. D., and P. A. L. Kongshavn.** 1987. Experimental murine tularemia caused by *Francisella tularensis*, live vaccine strain: a model of acquired cellular resistance. Microb. Pathog. **2:**3–14.
4. **Anthony, L. S. D., and P. A. L. Kongshavn.** 1988. H-2 restriction in acquired cell-mediated immunity to infection with *Francisella tularensis* LVS. Infect. Immun. **56:**452–456.
5. **Baron, G. S., and F. E. Nano.** 1998. MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. Mol. Microbiol. **29:**247–259.
6. **Buddingh, G. J., and F. C. Womack.** 1941. Observations on the infection of chicken embryos with *Bacterium tularense*, *Brucella* and *Pasteurella pestis*. J. Exp. Med. **74:**213–221.
7. **Chen, W. X., R. KuoLee, H. Shen, and J. W. Conlan.** 2004. Susceptibility of immunodeficient mice to aerosol and systemic infection with virulent strains of *Francisella tularensis*. Microb. Pathog. **36:**311–318.
8. **Clemens, D. L., B. Y. Lee, and M. A. Horwitz.** 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect. Immun. **72:**3204–3217.
9. **Conlan, J. W., A. Sjöstedt, and R. J. North.** 1994. $CD4^+$ and $CD8^+$ T-cell-dependent and -independent host defense mechanisms can operate to control and resolve primary and secondary *Francisella tularensis* LVS infection in mice. Infect. Immun. **62:**5603–5607.
10. **Cowley, S. C., C. J. Gray, and F. E. Nano.** 2000. Isolation and characterization of *Francisella novicida* mutants defective in lipopolysaccharide biosynthesis. FEMS Microbiol. Lett. **182:**63–67.
11. **Darby, C., C. L. Cosma, J. H. Thomas, and C. Manoil.** 1999. Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**15202–15207.
12. **Dienst, F. T.** 1963. Tularemia: a perusal of three hundred thirty-nine cases. J. La. State Med. Soc. **115:**114–127.
13. **Eigelsbach, H. T., and C. M. Downs.** 1961. Prophylactic effectiveness of live and killed tularemia vaccines. J. Immunol. **87:**415–425.
14. **Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz.** 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent *Francisella tularensis* infection. Infect. Immun. **12:**999–1005.
15. **Eigelsbach, H. T., J. J. Tulis, M. H. McGavran, and J. D. White.** 1962. Live tularemia vaccine. I. Host-parasite relationship in monkeys vaccinated intracutaneously or aerogenically. J. Bacteriol. **84:**1020–1027.
16. **Elkins, K. L., T. R. Rhinehart-Jones, S. J. Culkin, D. Yee, and R. K. Winegar.** 1996. Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. Infect. Immun. **64:**3288–3293.
17. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** Tularemia. Clin. Microbiol. Rev. **15:**631–646.
18. **Evans, M. E., D. W. Gregory, W. Schaffner, and Z. A. McGee.** 1985. Tularemia: a 30-year experience with 88 cases. Medicine **64:**251–269.
19. **Fortier, A. H., M. V. Slayter, R. Ziemba, M. S. Meltzer, and C. A. Nacy.** 1991. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. Infect. Immun. **59:**2922–2928.
20. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjöstedt.** 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect. Immun. **71:**5940–5950.
21. **Golovliov, I., A. Sjöstedt, A. Mokrievich, and V. Pavlov.** 2003. A method for allelic replacement in *Francisella tularensis*. FEMS Microbiol. Lett. **222:**273–280.
22. **Gray, C. G., S. C. Cowley, K. K. Cheung, and F. E. Nano.** 2002. The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. FEMS Microbiol. Lett. **215:**53–56.
23. **Hood, A. M.** 1977. Virulence factors of *Francisella tularensis*. J. Hyg. **79:**47–60.
24. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjöstedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J. Bacteriol. **186:**5808–5818.
25. **Kieffer, T. L., S. Cowley, F. E. Nano, and K. L. Elkins.** 2003. *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes Infect. **5:**397–403.
26. **Lai, X. H., I. Golovliov, and A. Sjöstedt.** 2001. *Francisella tularensis* induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. Infect. Immun. **69:**4691–4694.
27. **Larson, C. L., W. Wicht, and W. L. Jellison.** 1955. An organism resembling *P. tularensis* isolated from water. Public Health Rep. **70:**253–258.
28. **Lauriano, C. M., J. R. Barker, S.-S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassett, and K. E. Klose.** 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoeba and intramacrophage survival. Proc. Natl. Acad. Sci. USA **101:**4246–4249.
29. **Lindgren, H., I. Golovliov, V. Baranov, R. K. Ernst, M. Telepnev, and A. Sjöstedt.** 2004. Factors affecting the escape of *Francisella tularensis* from the phagolysosome. J. Med. Microbiol. **53:**953–958.
30. **Mariathasan, S., D. S. Weiss, V. M. Dixit, and D. M. Monack.** 2005. Innate immunity against *Francisella tularensis* is dependent on the ASC/caspase-1 axis. J. Exp. Med. **202:**1043–1049.
31. **Mdluli, K. E., L. S. Anthony, G. S. Baron, M. K. McDonald, S. V. Myltseva, and F. E. Nano.** 1994. Serum-sensitive mutation of *Francisella novicida*: association with an ABC transporter gene. Microbiology **140:**3309–3318.
32. **Moe, J. B., P. G. Canonico, J. L. Stookey, M. C. Powanda, and G. L. Cockerell.** 1975. Pathogenesis of tularemia in immune and nonimmune rats. Am. J. Vet. Res. **36:**1505–1510.
33. **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. M. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. I. Meierovics, G. Stephens, and K. L. Elkins.** 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J. Bacteriol. **186:**6430–6436.

34. **Nutter, J. E., and H. T. Eigelsbach.** 1967. Response of guinea pig to sublethal X-irradiation and live tularemia vaccine. Proc. Soc. Exp. Biol. Med. **124:** 1227–1230.
35. **Ransmeier, J. C.** 1943. The reaction of the chicken embryo to virulent and nonvirulent strains of *Bact. tularense*. J. Infect. Dis. **72:**86–90.
36. **Sandström, G., S. Löfgren, and A. Tärnvik.** 1988. A capsule-deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect. Immun. **56:**1194–1202.
37. **Schricker, R. L., H. T. Eigelsbach, J. Q. Mitten, and W. C. Hall.** 1972. Pathogenesis of tularemia in monkeys aerogenically exposed to *Francisella tularensis* 425. Infect. Immun. **5:**734–744.
38. **Telepnev, M., I. Golovliov, T. Grundstrom, A. Tarnvik, and A. Sjöstedt.** 2003. *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-α and IL-1 from murine macrophages. Cell. Microbiol. **5:**41–51.
39. **Vodovar, N., M. Vinals, P. Liehl, A. Basset, J. Degrouard, P. Spellman, F. Boccard, and B. Lemaitre.** 2005. *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. Proc. Natl. Acad. Sci. USA **102:**11414–11419.

Editor: D. L. Burns