Francisella Genes Required for Replication in Mosquito Cells

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J. Med. Entomol. 45(6): 1108–1116 (2008)

ABSTRACT *Francisella tularensis*, a potential bioterrorism agent, is transmitted by arthropod vectors and causes tularemia in many mammals, including humans. *Francisella novicida* causes disease with similar pathology in mice. We show that *F. novicida* invades hemocyte-like cells of the Sua1B cell line derived from *Anopheles gambiae* and replicates vigorously within these cells. We used transposon knockouts of single genes of *F. novicida* to show that bacterial growth within these insect cells is dependent on virulence factors encoded in a bacterial pathogenicity island that has been linked to replication in mammalian macrophages. The virulence factors MglA, IglA, IglB, IglC, and IglD as well as PdpA and PdpB were necessary for efficient growth in insect cells, but PdpC and PdpD were not required. The Sua1B cell line presents a valuable model to study the interactions between this important pathogen and insect vectors.

KEY WORDS *Francisella*, mosquito hemocyte-like cells, pathogenicity, virulence factor, vector biology

Francisella tularensis tularensis, the bacterium that causes tularemia, is a facultative intracellular pathogen that infects a broad spectrum of hosts (Ellis et al. 2002). Francisellae have been isolated from >250 species of vertebrates and from arthropod vectors including ticks, mosquitoes, and tabanid flies (Ellis et al. 2002, Sjostedt 2007). *Francisella* species are pathologic to fish (Birkbeck et al. 2007, Mauel et al. 2007, Mikalsen et al. 2007, Ottem et al. 2007), and related gamma proteobacteria occur as symbionts of ticks and other arthropods (Niebylski et al. 1997). Because of its high infectivity and potential for high morbidity and mortality, *F. tularensis* was weaponized during the 20th century by Japan, the United States, and the USSR. In 2002, it was designated by the CDC as a Category A Select Agent (Darling et al. 2002). Laboratory research on *Francisella* has used *F. tularensis* subspecies *tularensis* (the Category A Select Agent) as well as the less virulent *F. tularensis* subspecies *holarctica* (and its live vaccine strain [LVS]) and *F. tularensis* subspecies *novicida* (a.k.a. *Francisella novicida*), a strain with low virulence in humans (Hollis et al. 1989) but one that is very pathogenic in rodents.

Arthropod vectors have been implicated in the transmission of *Francisella* during multiple outbreaks of tularemia in both humans and animals (Skierska 1955, Christenson 1984, Morner et al. 1988, Hubalek et al. 1997, Eliasson et al. 2002). Although the classically identified vector was a deerfly (Francis 1922), the most commonly cited vectors in the United States have been ticks (Eisen 2007). Several epidemiological studies from Sweden point to a strong involvement of mosquitoes in the transmission of *F. t. holarctica.* In a case control study of a tularemia outbreak with 270 reported cases in Sweden in 2000, Eliasson and coworkers showed that the largest odds ratio of being infected was associated with mosquito bites (Eliasson et al. 2002). Most epidemics in Sweden occur during late summer and early autumn (Olin 1942, Tarnvik et al. 1996) during the active mosquito season and present in the ulceroglandular form of the disease that would be expected from arthropod transmission.

Vectors acquire *Francisella* through ingestion with a bloodmeal, and the bacteria can be found in large numbers within the cells of arthropods infected in the laboratory. In his 1927 paper on the model vectors *Dermacentor andersoni* and *Cimex lectularius*, Francis (1927) showed the bacteria within feces, the lumen of the gut, the midgut, and hindgut epithelia, the cells of the Malpigian tubules, the hemolymph, and (only in the ticks) the salivary glands. The drawings of Vyrostekova (1994) depict *F. t. tularensis* within hemocytes of ticks. The recent work of Aperis et al. (2007) showed that, after caterpillars of *Galleria mellonella* were inoculated with the *Francisella* LVS strain, the bacteria co-localize with hemocytes and kill the insect. Vonkavaara et al. (2008) infected adult *Drosophila* flies and cultured hemocytes with *Francisella* LVS. Although the *Drosophil*a mounted an immune defense, the bacteria proliferated and killed the flies and

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0022-2585/08/1108–1116$04.00/0 © 2008 Entomological Society of America

cultured *Drosophila* cells. The genetic adaptations of *Francisella* for life within arthropod cells have been little studied.

As a facultative intracellular bacterium in several mammalian cell types, *Francisella* is able to traverse intracellular compartments and to multiply. In mammalian macrophages, the bacteria are taken up into a membrane-bound phagosome (Anthony et al. 1991, Golovliov et al. 2003) that matures and acquires early and late endosomal markers. Within an hour, the bacteria escape the phagosome and replicate in the cytoplasm (Golovliov et al. 2003, Clemens et al. 2004, Santic et al. 2005, Checroun et al. 2006).

Seminal work on *F. novicida* in the laboratoratoy of Francis Nano at the University of Victoria (Canada) has identified several virulence factors that are required for growth of *Francisella* inside macrophages. In 1998, Baron and Nano identified the *mglAB* operon that encodes transcriptional regulators which are required for intracellular survival and growth (Baron and Nano 1998). The MglA protein controls the expression of 16–19 genes in a contiguous block designated as the francisella pathogenicity island (FPI) (Nano and Schmerk 2007). FPI genes include four intracellular growth loci (*iglABCD*) and four loci encoding pathogenicity determining proteins (*pdpABCD*). FPI gene products have been linked to escape from the phagosome and to replication in the cytoplasm (Nano and Schmerk 2007). It is possible that some of the virulence factors are generic for survival in any eukaryotic cell, whereas some others could be specific to the cellular environments in particular host or vector species. Published data for several bacteria that use arthropod vectors clearly indicate that there is extensive regulation of gene expression, both to enable the microbe to adapt to the arthropod environment and to prepare for transmission to the mammalian host (Schwan et al. 1995, de Silva et al. 1996, Kocan et al. 2002, Lohr et al. 2002, Grimm et al. 2004, Rovery et al. 2005).

In this paper, we present a model system for studying *Francisella*, using *F. novicida* and the Sua1B cell line from *Anopheles gambiae* (Dimopoulos et al. 1997). We show that *F. novicida* replicates within Sua1B and that *mglA* and at least six FPI genes (*iglABCD*, *pdpA*, and *pdpB*) are required for this replication. In concurrence with the recently presented data regarding the role of *pdpD* in mammalian macrophages (Nano and Schmerk 2007), we show that *pdpD* is not essential for replication within these insect cells. Furthermore, we show that *pdpC*, which was thought to be essential for replication in mammalian macrophages (Barker and Klose 2007), is not required for survival and replication in Sua1B hemocyte-like cells.

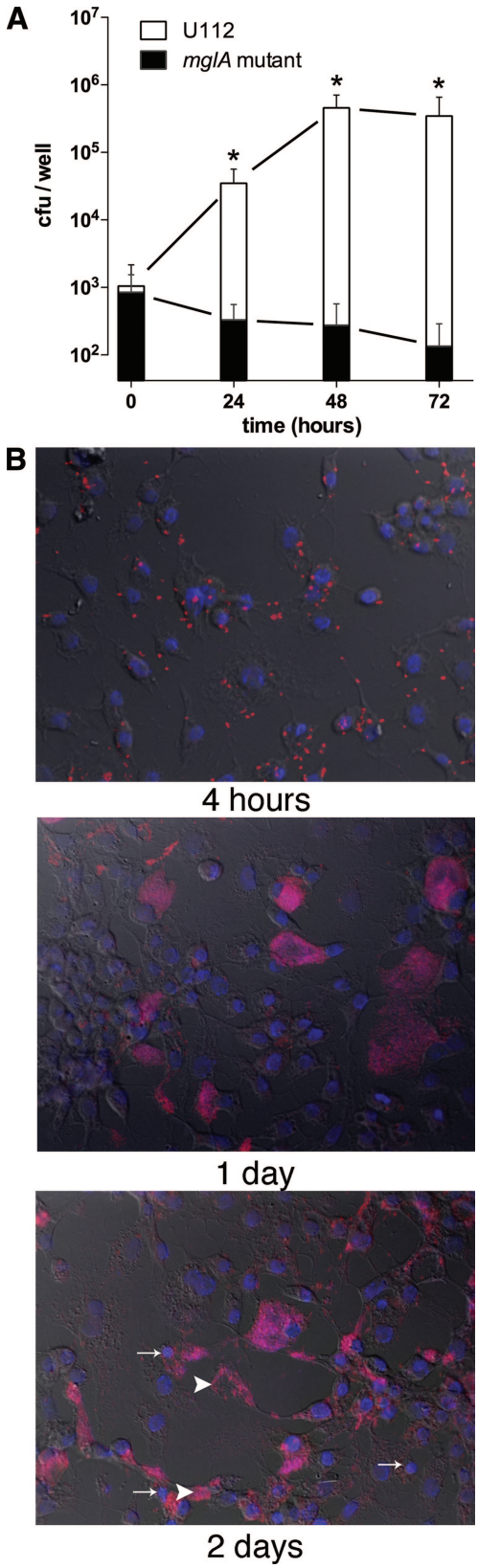
Materials and Methods

Bacterial Strains and Growth Conditions. *Francisella novicida* wild-type U112 was obtained from Colin Manoil and Larry Gallagher, University of Washington, the *mglA* (FN11 mutant strain) from Tina Guina, University of Washington, and the *pdpA* (f-box deletion mutant) from Francis Nano, University of Victoria, Victoria, Canada. Transposon insertion mutants were selected from the *F. novicida* transposon mutant collection (Gallagher et al. 2007). For each experiment, bacteria were grown overnight on tryptic soy agar plates supplemented with 0.1% L-cysteine and 0.2% glucose at 37°C.

Mosquito Cell Culture. The mosquito cell line Sua1B derived from *Anopheles gambiae* (Dimopoulos et al. 1997) was maintained in Schneider's *Drosophila* medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and incubated at room temperature.

Infection Assays. These assays were carried out according to Anthony et al. (1991) with modifications. After resuspension in fresh media, Sua1B cells were transferred to a 24-well plate at a concentration of 10^6 cells/well. Plates were incubated overnight at room temperature to allow for reattachment, and excess media were removed and discarded. *F. novicida* wild-type U112 and mutant strains were grown overnight on tryptic soy agar plates supplemented with 0.1% L-cysteine and 0.2% glucose at 37°C. Bacterial cells of each strain were resuspended in fresh media and added to the mosquito cell monolayers at a concentration of 10^8 cells/well followed by a 1-h incubation at room temperature to allow for uptake. All experiments were carried out at the same ratio of bacteria to cells, a multiplicity of infection (MOI) of 100. Excess bacteria were discarded and replaced with fresh media containing 500 μg/ml gentamicin. Plates were incubated for 2 h to kill extracellular bacteria. After 2 h (time point 0) and then in 24-h intervals to a maximum of 72 h, cells were lysed with 0.1% deoxycholate. The cell lysate was serially diluted in PBS or Schneider's *Drosophila* medium, plated onto tryptic soy agar plates supplemented with 0.1% L-cysteine and 0.2% glucose, and incubated overnight at 37°C. The number of bacterial colony forming units (CFUs) was determined retrospectively by counting the colonies on agar plates. Each time point was tested in triplicates, and assays were repeated twice. For each gene, two different mutant strains were used to ensure accuracy and avoid effects caused by strain-specific phenotypes (*Igl* mutant strains we used are listed in Fig. 3 and *pdp* mutant strains in Fig. 5).

Immunofluorescence. To assess replication of U112 by immunofluorescence, microscopy Sua1B cells were seeded on 18-mm glass coverslips in 24-well tissue culture plates at 2×10^5/well 1 d before infection. Cells were infected at a MOI of ~20 in 200 μl. The infected cells were centrifuged at 600*g* for 10 min to increase interaction between the bacteria and the mosquito cell line. Cells were incubated for 1 h at 28°C. Extracellular bacteria were removed by washing with Schneider's *Drosophilia* medium containing 10% FBS and gentamicin (50 μg/ml) and incubated in Schneider's *Drosophilia* medium containing 10% FBS and gentamicin. At the indicated times, the cells were fixed, permeabilized, and stained for *Francisella* using a rabbit anti-*F. novicida* antiserum (obtained from



Francis Nano, University of Victoria, Victoria, Canada) and Alexa Fluor 594–conjugated anti-rabbit antibodies (Invitrogen, Carlsbad, CA). DNA was stained using DAPI (Invitrogen) according to manufacturer's instructions. Images were collected in a Z-series on an Olympus IX81 microscope with a spinning disk confocal unit and analyzed using Slidebook software.

For inside-out staining, the extracellular bacteria were stained before fixation using the same primary antiserum as described above and Alexa Fluor 488–conjugated secondary antibodies. Subsequently all cell-associated bacteria were visualized after fixation and permeabilization and staining of all cell-associated bacteria using anti-novicida serum and Alexa Fluor 594–conjugated secondary antibodies.

Statistical Analysis. The data were analyzed by either unpaired, two-tailed *t*-test or one-way analysis of variance (ANOVA) with the Tukey post-test using GraphPad InStat software version 3.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

Results

***Francisella novicida* Infects Mosquito Cell Line Sua1B.** To study the suitability of the Sua1B hemocyte-like cell line as a model to study the *Francisella*–vector interaction on a cellular level, we infected Sua1B cells with *F. novicida* strain U112. A gentamicin protection assay was used to measure uptake and replication of bacteria inside mosquito cells. One million cells were incubated with *F. novicida* for 60 min, followed by addition of gentamicin to kill extracellular bacteria. At time point 0, which was taken 2 h after the addition of gentamicin, significant amounts of bacteria entered the Sua1B cells, now protected from the gentamicin in the growth medium. The number of CFUs recovered from lysed Sua1B cells increased over time, reaching a peak at 48 h after infection and then starting to decline by 72 h after infection (Fig. 1A). This clearly indicates that *F. novicida* is able to invade mosquito cells and to replicate within the cells.

To ensure that *F. novicida* did not survive in the growth medium, at each time point before the mos-

Fig. 1. *Francisella novicida* replicates within the mosquito Sua1B cell line. (A) Growth of wild-type U112 in Sua1B cells in comparison to the *F. novicida mglA* mutant over 72 h. Mosquito cells were infected with two different *mglA* strains at an MOI of 100. Time point 0 refers to 2 h after cells were treated with gentamycin. Data represent the means and SDs of at least two independent experiments for each strain carried out in triplicates. *Significant differences of the *mglA* mutants compared with the wild type at time points 24, 48, and 72 h after infection ($P < 0.05$ in unpaired, two tailed *t*-test). (B) Infected cells were fixed and stained for DNA and *F. novicida* after 4 h, 1 d, or 2 d of infection. The images were collected as a Z-series, projected to a single plane, and combined with a corresponding DIC image of the infected cells to show the outline of the Sua1B cells. Cells with pyknotic nuclei are indicated with arrows, and cells without a clearly visible nucleus are indicated by arrowheads.

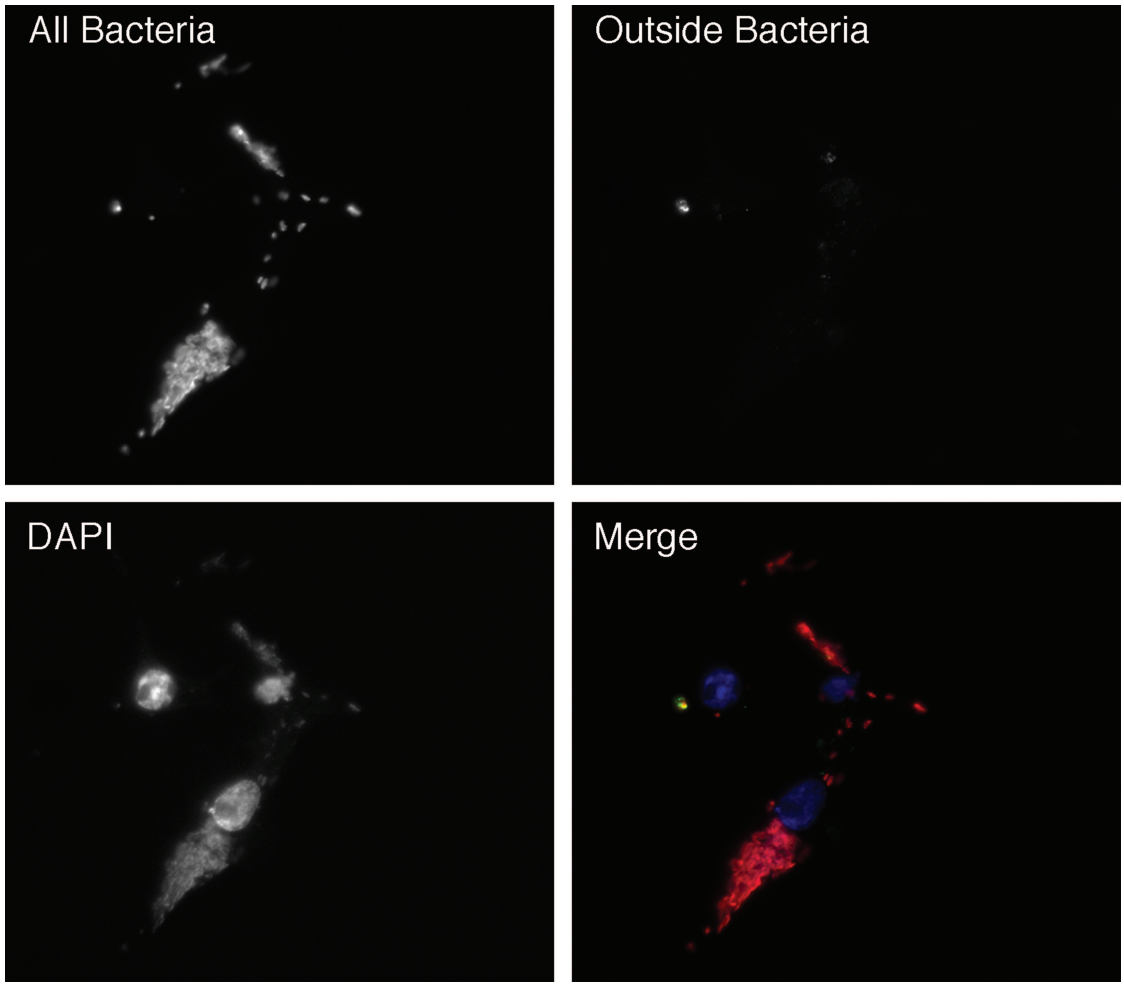


Fig. 2. Most cell-associated bacteria are intracellular. After 2 d of infection, differential staining of extracellular bacteria and all cell-associated bacteria shows the majority of bacteria within the cytoplasm of the infected Sua1Bcells. In the merged image, DNA is shown in blue, extracellular bacteria in green, and all cell-associated bacteria in red.

quito cells were lysed, 100 μl medium was taken of each well and plated. No CFUs were recovered from these wells (data not shown).

To visualize the replication of *F. novicida* in Sua1B cells, cells were subjected to immunofluorescence microscopy. In these experiments, infected cultures were fixed and stained for *Francisella* antigens and DNA at 4 h, 1 d, or 2 d after inoculation. As can be seen in Fig. 1B, the number of bacteria associated with the mosquito cells increases dramatically, especially within the first day of the infection. After 48 h, multiple cells had nuclei that appeared to be pyknotic or were weakly defined, indicating that the cells are stressed and are loosing their viability.

To ensure that the bacteria associated with the Sua1B cells are actually inside the host cells, we used both inside-out staining and three-dimensional reconstructions. The inside-out staining methods allows for differential labeling of bacteria on the outside of the cells, followed by staining of all bacteria within cells after permeabilization. As can be seen in Fig. 2, the majority of cell associated bacteria are not recognized by antibodies before permeabilization. The localization of bacteria within the infected arthropod cells was additionally confirmed by three-dimensional reconstruction of confocal images taken of infected cells 1, 24, and 48 h after infection (see supporting online material). These reconstructions also confirm the presence of *Francisella* inside the infected cells.

MglA, a Regulator for Virulence Genes in *Francisella*, Is Required for Replication of *F. novicida* Within Insect Cells. Multiple virulence factors have been described in *Francisella* that are necessary for infection of mammalian hosts and replication within macrophages or macrophage-like cell lines. To assess if these virulence factors are also required in this mosquito cell system, we examined the role of mglA, a regulator for a variety of virulence factors including several genes encoded by the FPI during

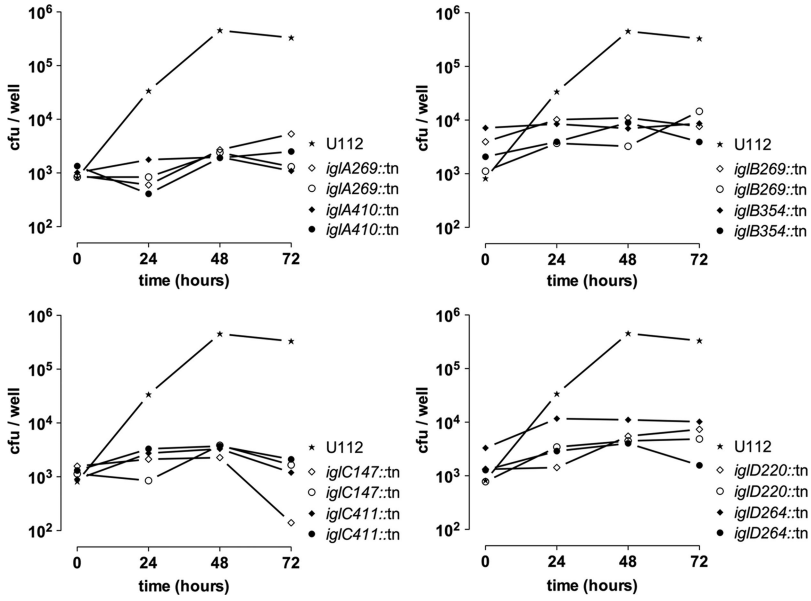


Fig. 3. Replication of *iglABCD* mutant strains in Sua1B cells over 72 h in comparison to the wild-type U112. Mosquito cells were infected with two different strains for each gene at an MOI of 100. Each experiment was carried out in triplicate.

cell infection. Mutants with transposon insertions in the *mglA* gene of U112 were able to enter the Sua1B cells with similar efficiencies compared with wild-type U112, because the number of recoverable CFUs at time point 0 was similar to wild-type bacteria (Fig. 1A). However, the *mglA* mutant bacteria did not replicate within Sua1B cells because the number of CFUs decreased over the following 3 d. These data indicate that some of the genes regulated by mglA are necessary for the replication of U112 in mosquito cells. The growth of the *mglA* mutants was statistically different from U112 ($P < 0.05$ in unpaired, two-tailed *t*-test) at time points 24, 48, and 72 h after infection.

Francisella Pathogenicity Island Genes Are Required for Efficient Replication of *F. novicida* in Sua1B Cells. To test if the proteins encoded by the FPI are necessary for replication of *Francisella* in Sua1B, we infected cell cultures with at least two strains carrying an independent mutant for each FPI gene, derived from the *F. novicida* transposon mutant collection library developed by Gallagher et al. (2007). We found that all four *igl* genes (*iglABCD*) were required for replication in these arthropod cells. Different mutant strains of individual *igl* genes were very similar in the lack of their ability to replicate (Fig. 3). We combined all data for each individual gene and expressed the data as relative increase of CFUs over time compared with the amount of bacteria that was present at time point 0. In comparison to the wild type, growth of all *igl* mutants was attenuated and statistically different (one-way ANOVA with Tukey posttest) after 48 ($P < 0.01$) and 72 h ($P < 0.05$; Fig. 4).

For each *pdp* gene, we tested two individual insertion mutants. Only *pdp*A and *pdp*B were required for replication. *pdp*A and *pdp*B mutants multiplied very slowly, showing <10-fold increases in CFUs over a 3-d period similar to the *igl* genes (Fig. 5). However, replication of *pdp*D and *pdp*C mutants in Sua1B cells was comparable to the wild type (Fig. 5). When data for each locus were combined and expressed as relative growth compared with initial CFU values, the differences between *pdp*A and *pdp*B mutants and the wild-type U112 again were statistically significant (one-way ANOVA with Tukey posttest) after 48 ($P < 0.01$) and 72 h ($P < 0.05$) of infection. The results for both *pdp*C and *pdp*D were not significantly different from wild-type bacteria (Fig. 6).

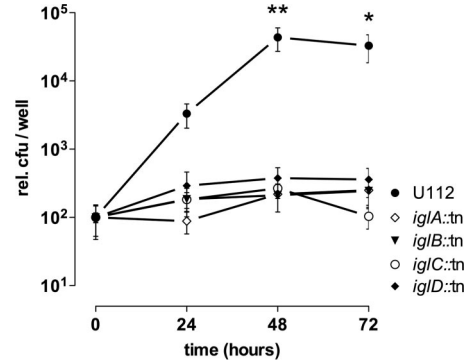


Fig. 4. Relative growth (CFUs) of *igl* mutant strains compared with the wild type. Data for each gene represent the averaged results from Fig. 2. Error bars indicate means ± SD. *Significant differences of all *igl* mutants compared with the wild type at time points 48 ($P < 0.01$) and 72 h ($P < 0.05$; one-way ANOVA with Tukey posttest).

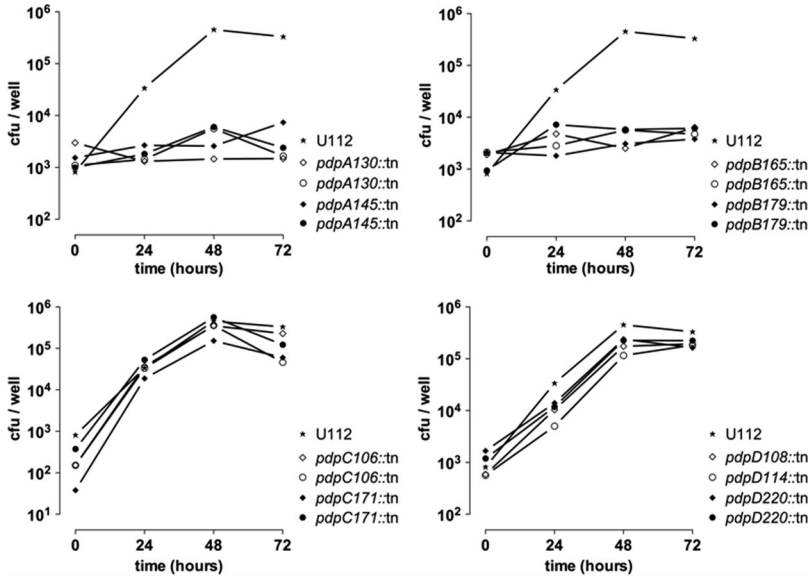


Fig. 5. Replication of *pdpABCD* mutant strains in Sua1B cells over 72 h in comparison to the wild-type U112. Mosquito cells were infected with two different strains for each gene at an MOI of 100. Each experiment was carried out in triplicate.

Discussion

We showed that *F. novicida* can infect and replicate in the Sua1B hemocyte-like cell line from the mosquito *An. gambiae.* Growth of *F. novicida* was rapid and extensive in the first 24 h after infection, and bacterial numbers continued to increase through 72 h. In macrophages, one of the preferred cell types during the infection of mammalian hosts, replication of *Francisella* causes cell death through apoptosis (Lai and Sjostedt 2003, Lai et al. 2001, Hrstka et al. 2005). Given the rate of replication observed in the mosquito cells in the early stages of infection, coupled with the loss of nuclear integrity and followed by cell death, we suggest that *Francisella* uses arthropod cells during infection of vector hosts in much the same way as macrophages in mammalian hosts. This conclusion is consistent with the drawings of Francis showing bacteria in the hemocoel of ticks and bed bugs (Francis 1927) and with the recent reports of *Francisella* growth in model systems such as *Galleria* and *Drosophila* (Aperis et al. 2007, Vonkavaara et al. 2008).

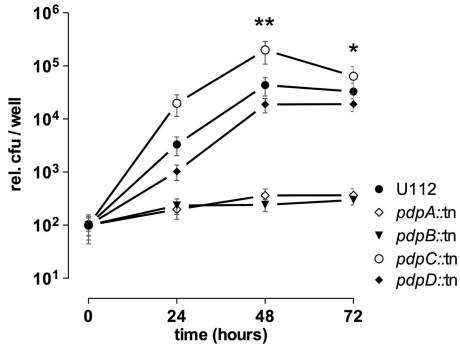


Fig. 6. Relative growth (CFUs) of *pdp* mutant strains compared with the wild type. Data for each gene represent the averaged results from Fig. 4. Error bars indicate means ± SD. *Significant differences of the mutants *pdpA* and *pdpB* compared with the wild type at time points 48 ($P < 0.01$) and 72 h ($P < 0.05$; one-way ANOVA with Tukey posttest).

The cellular roles and potential homologies of the *Francisella* virulence factors have been previously explored in mammalian cells and amoebae. The two gene operon, comprising *mglA* and *mglB*, is essential for intramacrophage growth of *Francisella* (Baron and Nano 1998). The product of *mglA* shows strong homology with SspA, a transcriptional activator associated with an *E. coli* RNA polymerase. SspA homologs have a role in virulence in other bacterial species. MglA acts as a transcriptional regulator for genes encoded in the FPI (Lauriano et al. 2004, Brotcke et al. 2006) as well as for potential virulence factors encoded outside the pathogenicity island (Brotcke et al. 2006). Mutants lacking functional *mglA* are defective for survival in mouse macrophages and the protozoan *Acantaoamoebae castellani* and have highly attenuated virulence in mice (Lauriano et al. 2004). In this study, the *F. novicida mglA* mutant was taken up by Sua1B cells but was unable to efficiently replicate in this mosquito cell line. This result suggested that, as for mammalian cells and amoebae, other genes regulated by *mglA*, including those in the FPI, are required for survival and replication in arthropod cells.

In 2004, Nano and coworkers showed that the FPI was essential for the survival and growth of *F. tularensis* within the macrophage (Nano et al. 2004). The FPI contains an operon comprised of four genes, *iglABCD*, upstream of which is an open reading frame designated *pdpD* and downstream three open reading frames, *pdpABC.* Only two genes (*iglA* and *iglB*) in this

genomic region encode products with any shown homology to those of other known genes (Nano et al. 2004). *iglA* and *iglB* are homologous to a locus encoding IcmF-associated homologous proteins (IAHPs) found in *Vibrio cholerae*, *Salmonella enterica*, and *Rhizobium leguminosarum* (Folkesson et al. 2002, Bladergroen et al. 2003, Nano et al. 2004, Sexton et al. 2004, de Bruin et al. 2007). In *Vibrio cholerae* and *Pseudomas aeruginosa*, members of the IAHPs have recently been implicated in a novel protein secretion system (type VI secretion) (Mougous et al. 2006, Pukatzki et al. 2006). These results led to the hypothesis that the FPI or parts of the FPI are involved in protein secretion and that this proposed secretion system is necessary for intracellular survival of *Francisella* in macrophages (de Bruin et al. 2007).

Some phenotypes have been assigned to mutants in different FPI genes. Insertion mutants in the genes encoding the FPI proteins IglC and IglA were not able to escape the phagosome and failed to replicate efficiently in mouse macrophages or macrophage-like cell lines (Gray et al. 2002, Lindgren et al. 2004, Santic et al. 2005, de Bruin et al. 2007). IglC and IglA mutants were also highly attenuated in a mouse infection model (Twine et al. 2005, de Bruin et al. 2007) and a chicken embryo model (Nix et al. 2006). *Francisella* strains lacking a functional gene encoding the FPI virulence factor IglD showed no defect in phagosomal escape but lower replication in the cytoplasm, indicating a different mechanism for IglD than for IglC and IglA within infected macrophages (Santic et al. 2007). In the mosquito Sua1B cell as for mammalian macrophages (Nano et al. 2004, Santic et al. 2005, 2007, de Bruin et al. 2007), all four *igl* genes are required for replication.

The disruption of the genes encoding PdpA or PdpB also showed reduced virulence in BALB/c mice (Nano et al. 2004, Tempel et al. 2006), and *pdpA* in particular has been shown to be important in bacterial escape from the phagosome (Mariathasan et al. 2005). Our results presented here indicate that these two genes are also important for replication of *F. novicida* in arthropod cells.

Barker and Klose stated that the *pdpD* gene is not required for growth of *Francisella* in mammalian macrophages (Barker and Klose 2007). When we examined the requirement for *pdpD* in Sua1 B arthropod cells, we obtained a similar result.

In contrast, Barker and Klose suggested that *pdpC* is important for growth of *Francisella* in mammalian macrophages (Barker and Klose 2007), but the preliminary supporting data were not presented. In this study, we found that *F. novicida* mutants deficient in PdpC were able to grow and survive in the Sua1B insect cell line at a level comparable with wild-type *F. novicida.* Further study is necessary to determine whether there is a difference in the requirement for PdpC between the mammalian and arthropod systems.

Our results in this study show the suitability of the Sua1B arthropod cell line as a model to investigate the role of potential *Francisella* pathogenicity factors. Apart from the finding that pdpC is not required for replication in Sua1B, the roles of the other tested FPI-encoded proteins seem consistent between arthropod and mammalian cells. Further studies are needed to establish the detailed mechanism of action as FPI encoded factors that facilitate bacterial invasion and growth in both mammalian and arthropod hosts. In addition, a better understanding of the *Francisella*–arthropod interaction is needed on a whole animal level to better understand arthropod immune response to *Francisella* ingested with a bloodmeal. The mosquito system offers attractive possibilities for future studies of the processing of *Francisella* within this model vector.

Acknowledgments

We thank F. Kafatos, Imperial College, London, and H.-M. Muller, EMBL, Germany, for donating Sua1B cells; T. Guina, University of Washington, and F. Nano, University of Victoria, Canada, for providing *F. novicida* U112 mutant strains; F. Nano, University of Victoria, Canada, for providing anti-novicida antiserum; and N. Gundlach, University of Alaska, for maintenance of the mosquito cell cultures. This project was supported in part by NIH NCRR INBRE Grant 2P20 RR016466 to the University of Alaska and a subcontract of NIH NIAID Research Center for Excellence in Biodefense (5U54AI057141) to the University of Washington.

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Received 1 May 2008; accepted 11 August 2008.