Disruption of *Francisella tularensis* Schu S4 *iglI, iglJ*, and *pdpC* Genes Results in Attenuation for Growth in Human Macrophages and *In Vivo* Virulence in Mice and Reveals a Unique Phenotype for *pdpC*


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*Francisella tularensis* is a facultative intracellular bacterial pathogen and the causative agent of tularemia. After infection of macrophages, the organism escapes from its phagosome and replicates to high density in the cytosol, but the bacterial factors required for these aspects of virulence are incompletely defined. Here, we describe the isolation and characterization of *Francisella tularensis* subsp. *tularensis* strain Schu S4 mutants that lack functional *iglI, iglJ, or dpdC*, three genes of the *Francisella* pathogenicity island (FPI). Our data demonstrate that these mutants were defective for replication in primary human monocyte-depended macrophages and murine J774 cells yet exhibited two distinct phenotypes. The *iglI* and *iglJ* mutants were similar to one another, exhibited profound defects in phagosome escape and intracellular growth, and appeared to be trapped in cathepsin D-positive phagolysosomes. Conversely, the *pdpC* mutant avoided trafficking to lysosomes, phagosome escape was diminished but not ablated, and these organisms replicated in a small subset of infected macrophages. The phenotype of each mutant strain was reversed by *trans* complementation. *In vivo* virulence was assessed by intranasal infection of BALB/c mice. The mutants appeared avirulent, as all mice survived infection with 10^8 CFU *iglJ*- or *pdpC*-deficient bacteria. Nevertheless, the *pdpC* mutant disseminated to the liver and spleen before being eliminated, whereas the *iglJ* mutant did not. Taken together, our data demonstrate that the pathogenicity island genes tested are essential for *F. tularensis* Schu S4 virulence and further suggest that *pdpC* may play a unique role in this process, as indicated by its distinct intermediate phenotype.

Tularemia is a potentially fatal illness caused by the facultative intracellular Gram-negative bacterium *Francisella tularensis* (1). Infection can occur following inhalation of as few as 10 bacteria, after ingestion of contaminated food or water, or, most commonly, after introduction of bacteria into the skin via the bite of infected arthropod vectors (2). The two subspecies of the organism that account for nearly all cases of human tularemia differ in geographic distribution and virulence, with highly virulent *Francisella tularensis* subsp. *tularensis* (type A strains) restricted to North America and less virulent *Francisella tularensis* subsp. *holoarctica* (type B strains) distributed throughout the Northern Hemisphere. An attenuated live vaccine strain (LVS) of *F. tularensis* subsp. *holoarctica* was developed several decades ago in the Soviet Union but is not currently licensed for use in the United States because the genetic and molecular explanations of attenuation are unknown (1). A related organism, *F. novicida*, causes a tularemia-like disease in mice but is avirulent in healthy humans (1, 3).

Macrophages are phagocytes that play a central role in immunity and host defense and that can also be used by certain pathogens, including *Francisella*, as vehicles for bacterial amplification and dissemination (1, 4). For this reason, many studies have focused on elucidating the *Francisella* life cycle in this cell type. Receptors that mediate phagocytosis of opsonized and unopsonized bacteria have been described and include complement receptor 3 (CR3) (CD11b/CD18), complement receptor 4 (CR4) (CD11c/CD18), scavenger receptor A, mannose receptor, and nucleolin (5–10). Shortly after uptake, both *tularensis* and *novicida* disrupt phagosome maturation so that phagosome-lysosome fusion is impaired and bacteria escape from a late-endosomal-like compartment to replicate in the macrophage cytosol (11–14). In this regard, it is noteworthy that LVS and all strains of virulent *F. tularensis* and *F. novicida* examined to date can replicate in primary human and murine macrophages and macrophage-like cell lines *in vitro* (4) despite the differences in host range and *in vivo* virulence noted above, and what accounts for this is unknown.

Central to *Francisella* virulence is a 30-kb segment of the genome called the *Francisella* pathogenicity island (FPI) that contains 16 to 19 predicted open reading frames organized in two large operons (15). The FPI was described first by Broms et al. and Nano et al. (15, 16) and is present in a single copy in *F. novicida*, yet it is duplicated in the genomes of all type A and type B *F. tularensis* strains, including LVS. Since *F. novicida* can be used in biosafety level 2 (BSL-2) laboratories, contains only a single copy of the FPI, and is more amenable to genetic manipulation than *F. tularensis* but still causes lethal disease in mice, the vast majority of studies to date have used the organism to define the roles of FPI genes in pathogenesis. Thus, it is established that the FPI regulatory factors MglA and FevR and at least some genes in the FPI, such as those within the intracellular growth locus (*iglABCD*) operon, are
ential for *Francisella* virulence in mice and are required for phagosome escape, intracellular growth, and blockade of phagosome maturation in macrophages (13, 16–19). These studies have been extended to LVS and human-pathogenic *F. tularensis* strains with similar results (20–22).

Other genes within the FPI are less well characterized. However, it has been proposed that pdpB, vgrG, dotU, iglI, and iglJ may encode a type VI-like secretion system, with iglI as a candidate secreted effector (17, 23). Nevertheless, the phenotypes of iglJ mutants generated in *F. novicida* and LVS are distinct, and explanations for these differences are lacking. Thus, whereas *F. novicida* iglI mutants are avirulent in mice and *Drosophila melanogaster* (17, 24, 25) and cannot escape the phagosome or replicate in J774A.1 macrophage-like cells in vitro (17, 23, 25, 26), this is not true for LVS iglJ mutants, as these organisms grow normally in J774 cells, although their replication was constrained to some extent in peritoneal exudate macrophages (26). The gene encoding IglJ is adjacent to and downstream of iglJ in the FPI. The phenotypes of *F. novicida* iglI and iglJ mutants are similar to one another, as strains lacking functional IglJ are also defective for phagosome escape and growth in both J774 cells and primary murine bone marrow-derived macrophages and do not cause disease in mice or flies (18, 23–25). We recently demonstrated that iglJ and iglI are not required for blockade of neutrophil NADPH oxidase activity by Schu S4 (27), but the effects of these genes on other type A *F. tularensis* virulence mechanisms have not yet been examined.

The FPI pathogenicity determinant protein C gene (pdpC) (15) is also of interest, as data in the literature are conflicting and argue both for and against a role for pdpC in regulation of *F. novicida* in vivo virulence, as well as bacterial growth in macrophages and mosquito cells in vitro (23–25, 28, 29). What accounts for these divergent findings is unclear. To date, the role of pdpC in type A *F. tularensis* virulence and intramacrophage interactions remains unexamined and awaits work to clarify these contradictory findings.

Previously, we reported the use of a transposon mutagenesis and screening strategy to identify genes in the virulent type A *F. tularensis* strain Schu S4 that are required for bacterial growth in human monocyte-derived macrophages (MDMs) (30). Although we did not expect to identify FPI genes in our mutagenesis strategy because of the FPI duplication, our results suggested that mutants with one functional and one disrupted copy of certain FPI genes may have subtle phenotypes. For this reason, and because the roles of iglJ, iglI, and pdpC in type A *F. tularensis* virulence were unknown, we sought to determine the fate of Schu S4 iglI, iglJ, and pdpC double-FPI-copy mutants in human MDMs and in mice after intranasal infection. Our results demonstrate that these FPI genes differentially contribute to Schu S4 virulence in vivo and in vitro. In particular, we report that the pdpC mutant has a distinct phenotype that differs from those of strains lacking functional iglI and iglJ and from most other FPI gene mutants characterized to date, as indicated by its ability to replicate in a subset of macrophages in vitro and its ability to disseminate in vivo before being eliminated by the host immune response.

**MATERIALS AND METHODS**

**Macrophage isolation and culture.** Heparinized venous blood was obtained from healthy volunteers following a protocol approved by the Institutional Review Board for human subjects at the University of Iowa. Peripheral blood mononuclear cells were isolated by density gradient separation with Ficoll-Hypaque; washed twice in HEPES-buffered, endotoxin-free RPMI 1640 medium (here called RPMI) supplemented with l-glutamine (both from Lonza, Walkersville, MD); and incubated in RPMI plus 20% autologous human serum at a concentration of 2 × 10^6 cells/ml in Teflon jars for 5 to 7 days at 37°C, 5% CO₂ for differentiation of monocytes into macrophages (31). MDMs were collected, washed with RPMI, counted on a hemocytometer, and plated according to experimental procedures described below. Replicate experiments were performed using cells from different donors.

**Bacterial growth and opsonization.** Frozen stocks of wild-type *F. tularensis* subsp. *tularensis* strain Schu S4 were used to streak cultures on one-day heart agar plates (DiCor, Sparks, MD) supplemented with 9% defibrinated sheep blood (Remel, Lenexa, KS), and cultures were grown for 2 days at 37°C, 5% CO₂. *Trans*-complemented mutant strains were grown on medium containing 25 µg/ml spectinomycin. Bacteria collected from agar plates were suspended and washed twice in Hanks’ balanced salt solution (HBSS) with divalent cations (HBSS2+ (Mediatech, Inc., Manassas, VA). Opsonization was performed by incubation of bacteria in 50% human serum (pooled from a minimum of 14 donors with no history of tularemia) for 30 min at 37°C, followed by washing three times in HBSS. All work with *F. tularensis* was performed in the University of Iowa Carver College of Medicine Biosafety Level 3 Facility in accordance with all Centers for Disease Control and Prevention guidelines. Experimental protocols were approved by the Carver College of Medicine Biosafety Level 3 Oversight Committee, and recombinant DNA work was approved by the Institutional Biosafety Committee.

**Construction of a pdpC mutant.** We previously reported generation of isogenic Schu S4 fevR, iglJ, and iglI mutants and *trans*-complemented strains (27). For this study, we again used group II intron retargeting (30, 32, 33) to disrupt pdpC in Schu S4. To this end, the Schu S4 pdpC-*sequence* was amplified for investigation using the Web-based Targeted Insertion (TIG) algorithm (Sigma-Aldrich, St. Louis, MO), and a site was selected for mutagenesis at nucleotide (nt) 608 of pdpC. A DNA fragment encoding a retargeted group II intron was generated by PCR and cloned into the XhoI and BsrGI sites of pKEK1140, and the plasmid was introduced into *F. tularensis* Schu S4 by cryotransformation (30). Transformants were initially grown on modified Mueller-Hinton (MMH) agar plates with 25 µg kanamycin at 30°C and then passed twice on nonselective MMH agar at 40°C to cure the temperature-sensitive plasmid. After confirmation of plasmid curing, clones were analyzed by PCR across the insertion site using primers CTATGGGGATTAGTTGATTTCGTAATAG and GCTTTGGACGACGTCGTGCGT and grown for 2 days on cysteine heart agar plates supplemented with 9% defibrinated sheep blood (Remel, Lenexa, KS), and cultures were grown for 2 days at 37°C, 5% CO₂. *Trans*-complemented mutant strains were grown on medium containing 25 µg/ml spectinomycin. Bacteria collected from agar plates were suspended and washed twice in Hanks’ balanced salt solution (HBSS) with divalent cations (HBSS2+ (Mediatech, Inc., Manassas, VA). Opsonization was performed by incubation of bacteria in 50% human serum (pooled from a minimum of 14 donors with no history of tularemia) for 30 min at 37°C, followed by washing three times in HBSS. All work with *F. tularensis* was performed in the University of Iowa Carver College of Medicine Biosafety Level 3 Facility in accordance with all Centers for Disease Control and Prevention guidelines. Experimental protocols were approved by the Carver College of Medicine Biosafety Level 3 Oversight Committee, and recombinant DNA work was approved by the Institutional Biosafety Committee.

**Reverse transcription (RT)-PCR.** RNA was isolated from bacteria grown for 2 days on cysteine heart agar plates supplemented with 9% defibrinated sheep blood using TRIzol Reagent (Invitrogen, Life Technologies, CA) following the manufacturer’s protocol. The RNA was treated with Ambion (Austin, TX) DNA-free rTaq DNA I, and cdNA was made following the standard protocol for the Applied Biosystems (Foster City, CA) high-capacity cDNA reverse transcription kit. cdNA was used for subsequent PCR with primers designed to detect a partial fragment (nucleotides 81 to 599) (GCATGATATTAAAAGAATCGAGGATGAC and GCTCTACAAAAATATGGAAGGA) or a full-length transcript (nucleotides 1503 to 1749) (TCCTCTGCTTCAGCTGCACTGCTGTG and AGCGATGTTGAGCGCAATATTGCCC) of pdpC. Primers CGGCTGCA ATTACAGAAAA and GGGCCAGGATCACATAC were used for pdpE.

**Sensitivity to lysis by serum complement.** Sensitivity to lysis by serum complement was tested by incubating bacteria (1 × 10^8/ml) in either 50% human serum or phos-
phosphate-buffered saline (PBS) (mock treated) for 90 min at 37°C with shaking. After serial dilution, the bacteria were plated for CFU enumeration.

Intramacrophage growth. MDMs were plated at 100,000 cells/well in 24-well tissue culture dishes in triplicate. The cells were allowed to adhere overnight in RPMI plus 10% human serum and then rinsed with warm PBS to remove nonadherent cells. Osponized bacteria were added to MDMs at a multiplicity of infection (MOI) of 20:1 in RPMI plus 10% human serum, and samples were incubated for 1 h at 37°C to allow bacterial uptake. After rinsing three times with PBS, the cells were incubated for 45 min at 37°C in fresh RPMI plus 2.5% human serum and 100 μg/ml gentamicin to inhibit the growth of any remaining extracellular bacteria. Subsequently, the wells were washed three times with PBS to remove the antibiotic, and RPMI plus 2.5% human serum was added to each well. Some cells were lysed at this time point (2 h postinfection [p.i.]) by addition of 1% saponin, and serial dilutions were spotted onto agar plates for CFU enumeration. Other samples were processed similarly at 20 to 26 h p.i. Infection of MDMs with unopsonized bacteria was performed in a similar manner, except that heat-inactivated human serum was used instead of fresh serum, and the MOI was increased to 100:1.

Bacterial growth in J774A.1 macrophage-like cells was also quantified. J774 cells were plated at 200,000 cells/well in antibiotic-free Dulbecco’s modified Eagle’s medium (DMEM) (Lonza) plus 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) and then infected with unopsonized bacteria at an MOI of 200:1. The initial infection efficiency and intracellular growth were quantified as described for MDMs.

For reinfection experiments, MDMs were infected at an MOI of 20:1 in RPMI containing 10% human serum. After 1 h, the cells were washed to remove free bacteria and returned to 37°C in medium supplemented with 2.5% human serum. Cell lysates prepared at 1 and 20 h p.i. were inoculated onto cysteine heart agar plates supplemented with 9% defibrinated sheep blood for CFU enumeration. Colonies from the 20-h samples were used to infect new MDMs, with quantitation of live intracellular bacteria at 1 and 20 h p.i.

Immunofluorescence microscopy. MDMs were plated at 40,000 cells/well in Nunc Lab-Tek glass chamber slides (Thermo Fisher Scientific, Rochester, NY) and then infected with opsonized bacteria at an MOI of 20:1 as described above. At 7.5 and 20 h p.i., cells were processed for microscopy using our established methods (21, 34). In brief, samples were fixed for 25 min at room temperature using 10% formalin (Sigma-Aldrich) and then permeabilized in 1:1 mixture of 20°C methanol-acetone for 15 min. The fixed and permeabilized cells were blocked at 4°C in PBS supplemented with 0.5 mg/ml NaN₃, 5 mg/ml bovine serum albumin (BSA), and 10% horse serum. Samples were then double stained to detect bacteria (using rabbit anti-F. tularensis antiseraum [BD Diagnostics]) and lysosome-associated membrane protein 1 (LAMP-1) [human anti-mouse MAB H4A3 from the Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA] or cathepsin D [MAB G-19; Santa Cruz Biotechnology, Santa Cruz, CA]). Dylight 488-conjugated AffiniPure F(ab’)₂ donkey anti-mouse and rhodamine-conjugated AffiniPure F(ab’)₂ donkey anti-rabbit secondary antibodies were from Jackson Immuno Research Laboratories (West Grove, PA). All samples were analyzed using a Zeiss LSM-510 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY). The number of infected cells and the number of bacteria per infected cell were determined after counting at least 100 infected cells within random fields of view. Colocalization of wild-type or mutant bacterial strains with LAMP-1 and cathepsin D was determined by analysis of at least 50 infected cells in triplicate samples at each time point. The data shown are the means and standard deviations (SD) from at least three independent experiments.

Phagosome integrity assay. Phagosome disruption and bacterial access to the cytosol were quantified using the method of Checrourn et al. with minor modifications (35). In brief, MDMs were plated in chamber slides and infected asynchronously with green fluorescent protein (GFP)-expressing wild-type or mutant bacterial strains as described above. At 1.5, 7.5, or 20 h p.i., 50 μg/ml digitonin in warm buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM MgCl₂, pH 7.3) was added for 1 min to selectively permeabilize the plasma membrane. The wells were then rinsed to remove residual digitonin, and anti-F. tularensis antiseraum was added for 12 min at 37°C to label bacteria with access to the cytosol. Cells were fixed and stained with rhodamine-conjugated F(ab’)₂ secondary antibodies. By this assay, all bacteria are GFP positive, whereas bacteria in disrupted phagosomes or free in the cytosol are both rhodamine and GFP positive. At least 100 infected cells for each bacterial strain and time point were analyzed, and the data shown are the means and SD from three independent experiments.

In vivo virulence and bacterial dissemination. Animal studies were performed using 6- to 8-week-old female BALB/c mice obtained from the National Cancer Institute (Frederick, MD). In preparation to inoculate the mice intranasally, each animal was anesthetized using intraperitoneal xylazine. Inocula were delivered in a total of 50 μl of PBS to each nare of an anesthetized mouse. Each strain was also diluted and plated onto agar plates to determine the actual number of CFU that were administered for each group of mice. Lethality studies utilized at least 5 mice per group. The mice were carefully monitored daily, and all animals showing signs of fatal disease were sacrificed. Dissemination was quantified by harvesting organs (lungs, liver, and spleen) from 3 mice per group on days 3, 5, 7, and 14 after infection. The organs were homogenized in PBS containing 3% saponin, and dilutions of each homogenate were spotted onto agar plates to quantitate the bacterial load in each organ by enumeration of CFU.

Statistical analysis. The statistical significance of data sets (P < 0.05) was determined using analysis of variance (ANOVA) with Dunnett’s or Tukey’s posttest for multiple comparisons, Student’s t test for paired samples, and log rank analysis using the Mantel-Cox test for analysis of Kaplan-Meier survival curves. All analyses were performed using GraphPad Prism 4 software.

RESULTS

F. tularensis Schu S4 iglI, iglJ, and pdpC mutants are defective for growth in primary human macrophages and murine J774A.1 cells. We previously reported the construction of a transposon mutant library that was used to identify genes in F. tularensis strain Schu S4 that are required for bacterial growth and survival in human MDMs (30). Schu S4 contains two identical copies of the FPI, and in our screen, we identified mutants that contained one intact and one disrupted copy of iglI or iglJ, although mutating only one of the two copies of these genes only marginally reduced Schu S4 growth in MDMs (reference 30 and data not shown). To better define the possible roles of these FPI genes in virulence of type A F. tularensis, we constructed Schu S4 iglI-153 and iglJ-189 mutants (referred to here as iglI and iglJ), as well as trans-complemented strains (referred to here as iglI⁺ and iglJ⁺) (27). In addition, we also constructed a Schu S4 pdpC-608 mutant (referred to here as pdpC) using a type II integron mutagenesis strategy (see Materials and Methods) (Fig. 1). Disruption of pdpC was confirmed by PCR (Fig. 1B). A complemented strain that expresses wild-type pdpC in trans (referred to as pdpC⁺) was also prepared. As disruption of pdpC at position 608 may have caused polar effects on the downstream gene pdpE or resulted in expression of a truncated (and perhaps partially functional) product, we also isolated bacterial RNA and used RT-PCR to assess gene expression. Primers were designed to amplify the region between nucleotides 81 and 599, which should be present in both truncated and full-length transcripts, as well as the region between nucleotides 1503 and 1749 that is specific for full-length pdpC, and a third set of primers was designed to detect expression of pdpE. The data in Fig. 1C show that Schu S4 expressed both pdpC and pdpE, as amplicons were generated using all three primer sets. In contrast, disruption of pdpC appeared to ablate expression of the gene, as we...
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found no evidence of full-length or truncated transcripts by RT-PCR. At the same time, expression of pdpE in the pdpC mutant strain appeared slightly diminished but was not ablated (Fig. 1C).

To characterize our mutants, we first determined if disruption of iglI, iglJ, or pdpC rendered the mutants sensitive to complement-mediated killing. To this end, bacteria were left in PBS or incubated in 50% pooled human serum and then plated for enumeration of CFU. By this assay, all three mutant strains were indistinguishable from wild-type Schu S4 and remained serum resistant (data not shown).

Next, we infected MDMs with serum-opsonized bacteria and quantified intracellular growth by enumeration of CFU at 2 and 20 h p.i., using wild-type Schu S4 and a fevR mutant as positive and negative controls, respectively. As shown in Fig. 2A, similar numbers of Schu S4 and the fevR, iglI, iglI^*, iglJ, iglJ^*, pdpC, and pdpC^+ strains were recovered from MDMs at 2 h p.i., indicating similar initial infection efficiencies. By 20 h p.i., Schu S4 had undergone more than 2 log_{10} growth, whereas the viability of the fevR mutant declined, confirming a defect in intramacrophage replication and survival (36). On the other hand, the numbers of viable iglI and iglJ mutant organisms recovered at 2 and 20 h p.i. were similar, indicating a significant defect in intracellular growth relative to Schu S4. Nevertheless, these strains were not eliminated, suggesting greater resistance to intracellular killing than the fevR mutant. In marked contrast to the other strains examined, the pdpC mutant appeared to undergo limited replication in MDMs, as the number of viable bacteria recovered increased 1.93-±1.68-fold between 2 and 20 h p.i. As recent data suggest that the mechanism of uptake can affect intracellular growth (37), we also infected MDMs with unopsonized bacteria and obtained similar results (Fig. 2B). Evidence that the mutations in the genes of interest were responsible for the phenotypes observed was obtained by trans complementation, although intra-MDM growth of the iglI^*, iglJ^*, and pdpC^+ strains did not reach wild-type levels (Fig. 2A and B). What accounts for this is unclear, but it is noteworthy that the complementation plasmid utilizes GroES rather than the native promoters, and the correct stoichiometry of FPI proteins is likely not achieved under these conditions.

As we have shown that a subset of Francisella mutants that are defective for growth in primary macrophages can still replicate in macrophage-like cell lines (38), we also quantified the replication of our unopsonized mutant strains in J774A.1 cells. These cells were infected to similar extents by all the strains examined; the pdpC mutant replicated to a limited extent (6.17-±3.75-fold) by 26 h p.i., whereas the other mutants did not; and intracellular growth was rescued by trans complementation of the iglI, iglJ, and pdpC mutants (Fig. 2C). Taken together, these data demonstrate that the FPI genes iglI, iglJ, and pdpC play important but perhaps distinct roles in intramacrophage growth of F. tularensis Schu S4.
The *pdpC* mutant replicates in a subset of infected macrophages. We used immunofluorescence microscopy to further characterize the intracellular phenotype and fate of mutant organisms by analysis of individual infected cells. To this end, MDMs were processed for confocal analysis at 7.5 and 20 h p.i. Pooled data from multiple experiments are shown in Fig. 3, and representative images of MDMs at 20 h p.i. are shown in Fig. 4 and 5.

At 7.5 h p.i. (Fig. 3A) nearly 40% of the Schu S4-infected MDMs contained 1 to 5 bacteria each. A similar fraction of infected cells contained 6 to 10 bacteria each, and the remainder of the cells contained 11 to 15 or >15 bacteria each, consistent with the onset of Schu S4 replication in MDMs by this time point (14, 30). In contrast, nearly all MDMs infected with mutant organisms contained only 1 to 5 bacteria. Results obtained for the *igII*, *igII+, igIj*, and *pdpC*+ strains were intermediate between those for wild-type and mutant organisms, as ~70 to 80% of infected cells contained 1 to 5 bacteria, 16 to 20% contained 6 to 10 bacteria, and 3 to 8% contained 11 to 15 bacteria. Consistent with the robust growth of wild-type *F. tularensis* in MDMs shown in Fig. 2A, a majority of Schu S4-infected MDMs contained more than 15 bacteria each by 20 h p.i. (Fig. 3B and 4), whereas results for the *fevR*, *igII*, and *igIj* mutants were essentially unchanged (Fig. 3B and 5).

In contrast, we observed two distinct phenotypes within the population of MDMs infected with the *pdpC* mutant (Fig. 4). Approximately 85% of these cells contained few bacteria (1 to 5 each) without evidence of intracellular growth and in this way resem-
bled MDMs infected with the other mutants examined in this study. Nevertheless, we also consistently observed a small subpopulation of infected MDMs (4.4% of the total) that contained large numbers of mutant bacteria that appeared to be replicating in the cytosol. Specifically, we found that 3.3% and 1.1% of MDMs infected with the \( \text{pdpC} \) mutant contained 11 to 15 bacteria or 15 bacteria, respectively, at 20 h p.i. (Fig. 3B, arrows). These results confirm and extend the data in Fig. 2, which indicate that some \( \text{pdpC} \) mutant organisms undergo limited replication in macrophages whereas the other mutant strains do not possess this ability. Although the data in Fig. 1C argue against expression of truncated, partially functional PdpC by our mutant, we also sought to determine whether our infection strategy may select for revertants or suppressor mutations that could also account for the atypical phenotype of the strain. To this end, MDMs were infected with the \( \text{pdpC} \) mutant; viable bacteria were recovered at 20 h p.i., quantified, and then used to reinfect new cells; and wild-type bacteria and the \( \text{iglI} \) mutant were used as controls. The data obtained for primary infection and reinfection were nearly identical for all strains tested (Fig. 3C) and are within the range of variability that we observe between different experiments. It is therefore highly unlikely that revertant or suppressor mutations account for the \( \text{pdpC} \) mutant phenotype.

Finally, our microscopy data also confirm the ability of \( \text{trans} \) complementation to restore rapid intracellular growth, as shown for \( \text{pdpC} \), \( \text{iglI} \), and \( \text{iglJ} \) strains in Fig. 3 to 5. Taken together, our CFU and microscopy data demonstrate that FPI genes \( \text{iglI} \), \( \text{iglJ} \), and \( \text{pdpC} \) are required for growth of type A \( \text{F. tularensis} \) in MDMs and that disruption of \( \text{pdpC} \) results in a phenotype that differs from those of mutant organisms lacking functional \( \text{iglI} \), \( \text{iglJ} \), or \( \text{fevR} \).

**Distinct roles of PdpC, IgII, and IgJ in phagosome escape.** To determine if the intramacrophage growth defects we observed could be attributed to defects in phagosome escape, we quantified phagosome integrity using an assay based on the method of Checroun et al. (35). By this assay, 50% of live, wild-type Schu S4 bacteria had access to the cytosol by 90 min postinfection (Fig. 6A). In contrast, only low-level background staining was detected when cells were infected with killed Schu S4 bacteria or when digitonin was omitted (13% cytosolic bacteria) (Fig. 6). Under the same conditions, the ability of the \( \text{iglI} \), \( \text{iglJ} \), and \( \text{fevR} \) mutants to breach the phagosome membrane was markedly impaired (\( P < 0.01 \) versus live Schu S4), and access of these organisms to the cytosol was increased by only 5 to 7% over the background obtained for the negative controls (Fig. 6), which was not statistically significant. Of note, our results for killed Schu S4 and the \( \text{fevR} \) mutant are in agreement with published data (14, 39).
Moreover, the extent of phagosome escape we report for strains lacking functional iglI and iglJ is nearly identical to that for a Schu S4 AiglC mutant (14). In marked contrast, the ability of the pdpC mutant to breach the phagosome membrane and reach the cytosol was diminished but not ablated. Thus, at 90 min postinfection, ~35% of the pdpC mutants were cytosolic, which represents a statistically significant increase ($P < 0.01$) relative to the killed Schu S4 control. We therefore conclude that PdpC contributes to phagosome escape, in contrast to IgI, IgJ, and FevR.

Enhanced colocalization of mutant organisms with LAMP-1. Next, we quantified the extent of bacterial colocalization with LAMP-1, a glycoprotein that is abundant in the membranes of late endosomes and lysosomes (11, 19, 21). At 7.5 h p.i., only ~15% of wild-type bacteria colocalized with LAMP-1, in keeping with published findings (11, 14, 19) and the data shown in Fig. 3A and 6. In contrast, we and others have previously reported that fevR mutants in F. tularensis LVS and Schu S4 are defective for phagosome escape and are trapped in LAMP-1-positive compartments (21, 36), and we confirm these data here (Fig. 7A and B). In addition, we extended these results to show that colocalization of the iglI, iglJ, and pdpC mutants, but not the complemented strains, with LAMP-1 was also significantly enhanced relative to the wild type (Fig. 7A and B).

IglI and IglJ are required to prevent phagosome-lysosome fusion. Previous studies have shown that wild-type F. tularensis escapes from a LAMP-1-positive and cathepsin D-negative compartment to replicate in the macrophage cytosol (11), and some regulator or FPI gene mutants that are defective for phagosome escape are also unable to prevent phagosome-lysosome fusion (13, 21, 40). Therefore, we also quantified phagosome maturation by analyzing the extent of mutant colocalization with cathepsin D. As expected, only about 10% of Schu S4 colocalized with cathepsin D at 7.5 h p.i. (Fig. 8A and B). In contrast, colocalization with this lysosomal enzyme was moderately but significantly increased by disruption of fevR and more profoundly affected by disruption of iglI or iglJ (Fig. 8A and B). Colocalization of the pdpC mutant with cathepsin D was also enhanced but did not reach statistical significance ($P > 0.05$) when all samples were analyzed in parallel using ANOVA (Fig. 8A and B). A similar pattern was obtained when each mutant was compared to the wild type by Student’s $t$ test, though in this case the results for the pdpC strain did reach statistical significance: $P = 0.0001$ for iglI, $P = 0.0016$ for iglJ, $P = 0.0022$ for fevR, and $P = 0.0185$ for pdpC. In contrast, the complemented strains resembled wild-type Schu S4. These data strongly suggest that only a subset of FPI genes are essential to prevent F. tularensis trafficking to lysosomes and that IgI and IgJ play critical roles in this process, whereas PdpC may not.

pdpC and iglJ mutants are attenuated for virulence in vivo, despite dissemination of bacteria lacking PdpC. Finally, we performed intranasal infections of BALB/c mice to evaluate the contributions of IgI and PdpC to in vivo virulence. In our hands, mice succumbed to infection with wild-type Schu S4 within 6 days after inoculation with 53 or 1,666 CFU (Fig. 9A). The virulence of the iglI and pdpC mutants was examined by challenging mice in parallel with approximately $10^7$ to $10^8$ CFU. Despite the high inocula, all mice remained healthy, did not show signs of disease, and were alive at day 14 after infection. These data extend our in vitro find-
By day 5, all Schu S4-infected mice had succumbed to infection or were euthanized due to moribund status. In contrast, none of the mice infected with either the iglI or pdpC mutant displayed signs of disease through day 14. Despite this, the iglI mutant was detected in the lung, but not the liver or spleen, on days 5 and 7 postinfection. On the other hand, the pdpC mutant was recovered in even greater numbers from the spleen on day 5 than on day 3 and persisted in the lung yet was cleared from the liver. The pdpC mutant was also recovered from mouse lungs and spleens on day 7, though bacterial burdens in these tissues had begun to decline, and by day 14 both mutants had been cleared from all three tissues (Fig. 9B and data not shown). The ability of the pdpC mutant to disseminate, particularly to the spleen, contrasts with the fate of the iglI strain and is consistent with the phenotypic differences that we observed in vivo. A role for PdpC in F. tularensis virulence in vivo was further assessed by infecting mice with 500 CFU of the complemented pdpC+ strain, and all five animals succumbed by day 5 or 6 (data not shown). Considered together, the results of this study indicate that although both IgI and PdpC are required for virulence, the ability of the pdpC mutant to replicate to a limited extent in murine organs in vivo, and in murine and human macrophages in vitro, indicates that disruption of pdpC in fully virulent type A F. tularensis results in a previously unappreciated and distinct phenotype compared with other FPI mutants examined to date.

**DISCUSSION**

Significant research efforts over the past decade have focused on understanding the molecular and genetic features responsible for the pathogenesis of tularemia. These studies have utilized isolates of several different Francisella species and subspecies, including F. novicida strain U112, F. tularensis subsp. holarctica LVS, and F. tularensis subsp. tularensis strain Schu S4, and have examined the fates of these organisms in insect and mammalian cells and in animal models. Each of these experimental systems has both desirable and undesirable aspects. Several seminal discoveries in the field were made using F. novicida, and this was facilitated by the relative ease of genetic manipulation of the organism, its ability to cause a tularemia-like disease in mice, and the ability to perform experiments without the precautions and containment required for studies of select agent strains of F. tularensis (23, 41–43). LVS has some of the same advantages, but its mechanism of attenuation is not defined, and neither F. novicida nor LVS causes disease in healthy humans, despite retaining the ability to replicate in human macrophages in vitro (3). A major challenge remains in the field to identify and characterize genes and other traits that account for the broad host range of the organism in general and the pronounced virulence of type A F. tularensis strains in humans. It is possible that individual Francisella genes may affect virulence in one host or cell type but not another. Mouse models are attractive due to the powerful genetic tools available; however, it should be noted that murine and human macrophages are not identical in their cellular physiology, especially in response to pathogenic microbes (44, 45). The results of this study demonstrate that FPI genes, iglI, iglJ, and pdpC, differentially contribute to virulence of the human-pathogenic type A F. tularensis strain Schu S4.

In our hands, disruption of either iglI or iglJ in Schu S4 had no effect on bacterial uptake by MDMs or J774 macrophage-like cells in the presence or absence of opsonins yet ablated intracellular...
At the cellular level, both mutants had profound defects in phagosome escape, as judged by differential staining, and were unable to prevent phagosome maturation, as indicated by their enhanced colocalization with cathepsin D, as well as LAMP-1. These data are consistent with studies by two groups that reported defective replication of \textit{F. novicida }\textit{iglI} mutants in murine bone marrow-derived macrophages (17) and J774 cells (23) and are also in keeping with a model in which \textit{IglI} is translocated into the macrophage cytosol by a type VI-like secretion system encoded within the FPI (17). However, a different phenotype has been reported for \textit{iglII} mutants generated in the LVS background (26). This \textit{DeltaiglII} mutant is defective for growth in murine peritoneal exudate macrophages but not in J774 cells by CFU analysis, although, interestingly, it exhibited increased colocalization with LAMP-1 and impaired phagosome escape, as judged by transmission electron microscopy (26). Also, in contrast to our findings and to parallel studies of \textit{iglI} in this organism, deletion of \textit{iglII} in \textit{F. novicida} has little or no effect on bacterial growth in J774 cells, after correcting for diminished uptake relative to the wild type (23). What accounts for these diverse phenotypes is unclear, as they cannot be attributed to the FPI copy number, the macrophage species, the diminished microbicidal capacity of cell lines, or uptake in the presence or absence of opsonins, and we extended our in vitro studies to show that \textit{iglJ} is also required for \textit{Schu S4} virulence in a mouse intranasal-infection model. We therefore conclude that \textit{iglI} and \textit{iglJ} are essential for \textit{Schu S4} virulence. In future studies, it will be important to elucidate the specific functions of \textit{IglI} and \textit{IglJ} with a focus on bacterial fate in macrophages.
and to determine whether their concerted actions are particularly important for the extreme virulence of type A F. tularensis.

The literature regarding the role of pdpC in Francisella virulence is also complex and somewhat contradictory. A report from Weiss et al. used an F. novicida transposon mutagenesis library to identify genes that were negatively selected in a mouse intraperitoneal-infection model and identified, in addition to pdpC, all FPI genes, including iglJ and iglI, as required for in vivo virulence (24). de Bruin et al. also found that pdpC is required for in vivo virulence of F. novicida yet at the same time reported that the gene is dispensable for bacterial replication in J774 cells (23). In contrast, Barker and Klose reported that pdpC is important for F. novicida growth in the same cell type, though the data were not shown in the publication (42). Additional studies in insects also obtained divergent results. Thus, one screen identified pdpC as being required for virulence of F. novicida in D. melanogaster (29), whereas another, similar screen identified all FPI genes as important except pdpC, pdpE, pdpD, and ammK (25). pdpC is also dispensable for F. novicida infection of Sua1B cells, which are derived from the Anopheles gambiae mosquito (28). Altogether, these data suggest that, for at least F. novicida, pdpC may play a role in virulence under certain conditions and/or during infection of cell types other than macrophages.

FSC043 is a spontaneous Schu S4 variant that is attenuated in mice (20). Subsequent analysis revealed that the FSC043 strain contains four insertions/deletions not present in Schu S4 in FTT0615 and FTT0918/FTT0919, as well as single-base-pair deletions in each copy of pdpC—FTT1354 (pdpC1) and FTT1709 (pdpC2) (46). Disruption of pdpC and the other affected genes in this manner likely introduced frameshift mutations, and growth of the FSC043 strain in peritoneal exudate macrophages is markedly impaired compared to Schu S4, but the relative contribution of each mutation to this phenotype is unknown (20).

For this study, we generated a pdpC mutant in F. tularensis Schu S4. Our mutagenesis strategy appeared to ablate pdpC, expression of the downstream gene pdpE was only slightly diminished, and we report subtle yet significant in vitro phenotypic deficits in the strain that are consistent with the results of our in vivo studies. Specifically, our data indicate that loss of functional pdpC diminished but did not ablate phagosome escape and that the mutant organisms replicated in a small subset of infected macrophages. As ~35% of these organisms reached the cytosol yet growth occurred in only ~4% of infected cells, our results indicate that phagosome escape is necessary but not sufficient for replication of the mutant in MDMs. At the same time, our data also suggest that pdpC may not be essential for the blockade of phagosome-lysosome fusion, as mutants that did not reach the cytosol resided in LAMP-1-positive compartments but did not exhibit markedly enhanced colocalization with cathepsin D, unlike strains lacking functional iglJ or iglI. These data are consistent with the idea that dissolution of the phagosome membrane and inhibition of phagosome maturation are distinct aspects of the Francisella life cycle in host cells and as such may be controlled by different subsets of virulence factors. Moreover, the distinct pdpC mutant phenotype we report here could easily have been overlooked if bacterium-macrophage interactions had been analyzed only by measurement of CFU or if our in vivo studies had not included quantitation of bacterial dissemination to the liver and spleen. It is also noteworthy that in vivo virulence was restored by complementation with a wild-type copy of pdpC expressed in trans. These data are strong evidence that the phenotypes we report are predominantly, if not exclusively, attributable to disruption of pdpC rather than diminished expression of pdpE. As at least some capacity for intracellular and in vivo growth appears to be essential for generation of protective immunity to the organism (20, 47), it will be of interest in future studies to determine whether pdpC-deficient strains of type A F. tularensis may be effective vaccines.

The function of PdpC is unknown. Kinetic analyses of F. tularensis gene expression in murine macrophages suggest that pdpC is downregulated at all time points examined between 1 and 24 h after infection, whereas expression of all other FPI genes is enhanced, albeit to different extents (36). As judged by immunoblotting, PdpC is relatively stable, whereas IgIc increased sharply during the first 4 h of infection (36). These data suggest that pdpC expression is regulated differently than that of other FPI genes, and our findings suggest that PdpC may play a distinct role in virulence. These data raise several questions. Specifically, what are the events that allow replication in such a small subset of pdpC mutant-organism-infected cells? Is there a unique subset of macrophages that support this outcome? To what extent is PdpC required for F. tularensis growth in other cell types, including dendritic cells, epithelial cells, and neutrophils? Perhaps more interesting will be to further characterize the fate of the pdpC mutant in vivo by examining what cell populations it is able to infect and how the organism disseminates.

In summary, the results of this study demonstrate that iglJ, pdpC, and iglJ are all required for intramacrophage growth of the type A F. tularensis strain Schu S4. IgIc and IgIj are essential for phagosome escape and blockade of bacterial trafficking to lysosomes in MDMs. In contrast, PdpC contributes to, but is not essential for, disruption of the phagosome membrane, and pdpC mutants replicate in a subset of infected macrophages in vitro. Moreover, the pdpC mutant disseminates from the lung to the liver and spleen in mice before being eliminated in vivo, whereas bacteria lacking functional iglJ do not. Our findings are noteworthy, as the phenotype we report for pdpC is atypical relative to other FPI genes examined to date and because we define potentially important differences between human-pathogenic type A F. tularensis strains, F. novicida, and LVS.

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