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Acid Phosphatases Do Not Contribute to the Pathogenesis of Type A *Francisella tularensis*▽†

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The intracellular pathogen *Francisella tularensis* is the causative agent of tularemia, a zoonosis that can affect humans with potentially lethal consequences. Essential to *Francisella* virulence is its ability to survive and proliferate within phagocytes through phagosomal escape and cytosolic replication. *Francisella* spp. encode a variety of acid phosphatases, whose roles in phagosomal escape and virulence have been documented yet remain controversial. Here we have examined in the highly virulent (type A) *F. tularensis* strain Schu S4 the pathogenic roles of three distinct acid phosphatases, AcpA, AcpB, and AcpC, that are most conserved between *Francisella* subspecies. Neither the deletion of *acpA* nor the combination of *acpA*, *acpB*, and *acpC* deletions affected the phagosomal escape or cytosolic growth of Schu S4 in murine and human macrophages, despite decreases in acid phosphatase activities by as much as 95%. Furthermore, none of these mutants were affected in their ability to cause lethality in mice upon intranasal inoculation. Hence, the acid phosphatases AcpA, AcpB, and AcpC do not contribute to intracellular pathogenesis and do not play a major role in the virulence of type A *Francisella* strains.

The Gram-negative bacterium *Francisella tularensis* is a highly infectious, facultative intracellular pathogen that causes tularemia, a widespread zoonosis affecting humans. Human tularemia is a fulminant disease that can be contracted by exposure to as few as 10 bacteria, the pneumonic form of which can lead to mortality rates as high as 25% if untreated (35). Three subspecies of *F. tularensis*, *Francisella tularensis* subsp. *tularensis* (type A), *Francisella tularensis* subsp. *holarctica* (type B), and *Francisella tularensis* subsp. *mediasiatica*, are recognized, among which strains of the first two subspecies can cause tularemia in humans (15). While type B strains are geographically distributed all over the northern hemisphere, the highly virulent type A strains are restricted to North America and account for the most-severe cases of the disease. *Francisella novicida*, a species of low virulence in humans but high virulence in rodents, has been used extensively as a surrogate model of *F. tularensis* pathogenesis, based on the assumption that it uses conserved virulence mechanisms (4, 7, 8, 19, 23, 25–29, 31, 41–45, 47). As a facultative intracellular pathogen, *F. tularensis* is capable of infecting and proliferating in a variety of host cell types, including hepatocytes, epithelial cells, and mononuclear phagocytes (15). Macrophages constitute an important target for infection in vivo (21), and the pathogenesis of *F. tularensis* depends on the bacterium's ability to survive and replicate within these host cells (15). Upon phagocytosis, *Francisella* ensures its effective survival and proliferation via rapid phagosomal escape followed by extensive replication in the cytosol (11, 14, 20, 42), thereby segregating itself from the degradative endosomal system and its associated bactericidal activities. Phagosomal escape is a tightly regulated process whose efficiency depends on conditions encountered within the early phagosome (12, 41), such as vacuolar acidification, although some controversy remains as to whether *Francisella*-containing phagosomes are significantly acidified prior to membrane disruption (13). Regardless of such discrepancies, phagosomal escape is an essential step in *Francisella* intracellular pathogenesis, since it is a prerequisite for cytosolic replication. Indeed, *Francisella* mutants that are defective in phagosomal escape do not grow intracellularly and are attenuated in vivo (6, 24, 43–45), and a belated phagosomal escape delays intracellular proliferation of the highly virulent type A strain Schu S4 (12).

Much effort has focused on identifying bacterial factors that contribute to phagosomal escape. Several genes located within a 30-kb chromosomal locus known as the *Francisella* pathogenicity island (FPI) (31) are required for proper phagosomal escape of *F. novicida* (43, 44) and the attenuated *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) (6, 24), since transposon insertions or targeted deletions in *iglC*, *iglD*, and *pdpA* affect the translocation of the mutants to the cytosol. Based on the homology of some FPI proteins with components of type VI secretion systems in other pathogens (30, 36), the FPI likely encodes a secretion apparatus that is required for phagosomal disruption. Yet a true understanding of FPI functions and the characterization of actual *Francisella* effectors of phagosomal escape are lacking. In addition to the FPI, Mohapatra et al. have recently reported for *F. novicida* that the acid phosphatases AcpA, AcpB, AcpC, and Hap are required for phagosomal escape and virulence in mice (27, 29). Acid phosphatases, which are ubiquitous in nature and hydrolyze phosphomo-

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noesters at acidic pHs, have been associated with the survival of intracellular parasites within phagocytes through inhibition of the respiratory burst (1, 3, 9, 22, 37–40), suggesting that they act as virulence factors. In *Francisella*, a prominent role was established for AcpA, an unusual, respiratory-burst-inhibiting enzyme exemplifying a novel family of acid phosphatases (18, 37). AcpA accounts for most of the acid phosphatase and phospholipase activities in the outer membrane fraction of *F. novicida* (29). These reports assigned acid phosphatases a role in phagosomal escape yet contradicted a previous study by Baron et al., who concluded that AcpA was not required for the intracellular growth or virulence of *F. novicida* (4). While the *acpA* mutants were constructed differently in these studies, the acid phosphatase activity associated with AcpA was abolished in both situations. A proposed explanation for these conflicting results was that the truncated AcpA generated by Baron et al. remained functional as a phospholipase C (37), an activity that would be required for phagosomal escape and virulence (27). Yet this hypothesis has not been tested, leaving the role of AcpA in *Francisella* virulence a controversial matter.

All studies of *Francisella* acid phosphatases have been carried out with *F. novicida* (4, 27, 29, 37), raising the question of significance with regard to the virulent *F. tularensis* subspecies. In particular, recent whole-genome comparisons between *F. novicida and* the different *Francisella tularensis* subspecies have highlighted important intervening sequence (IS)-mediated genome rearrangements in *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* strains relative to *F. novicida* (10). Such rearrangements have disrupted large numbers of open reading frames (ORFs), thereby creating pseudogenes (10) and likely inactivating many functions in virulent *F. tularensis* strains. For example, Mohapatra et al. (29) have reported that the virulent type A strain Schu S4 is missing a homolog of one of the two *hap* genes (FTN_0022) present in *F. novicida*, raising the question of conservation of acid phosphatase-encoding genes in virulent strains. Because phagosomal escape is an essential stage of the *Francisella* intracellular cycle that is common to *F. novicida* and *F. tularensis*, we have postulated that factors required to promote this process must be conserved between these organisms. Here we have compared acid phosphatase-encoding genes in *F. novicida* and virulent *F. tularensis* subspecies, and we have generated deletion mutants of the most conserved genes in Schu S4 in order to test their role in the phagosomal escape and pathogenesis of the highly virulent *F. tularensis* subspecies. We demonstrate that most acid-phosphatase-encoding genes are disrupted in virulent strains and that the most conserved loci are not required for phagosomal escape and virulence.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The prototypic type A virulent strain, *F. tularensis* subsp. *tularensis* Schu S4, was obtained from Rick Lyons (University of New Mexico, Albuquerque, NM). The Schu S4 Δ*fevR* mutant (ΔFTT0383) has been described previously (46). Bacteria were grown on modified Mueller-Hinton (MMH) agar plates (Mueller-Hinton medium supplemented with 0.1% glucose, 0.025% ferric pyrophosphate, and 2% IsoVitaleX [Becton Dickinson, Cockeysville, MD]) for 3 days at 37°C under 7% CO_2. For allelic replacement, MMH medium was supplemented either with 10 μg/ml kanamycin (Invitrogen, Carlsbad, CA) or with 8% sucrose (Sigma, St Louis, MO). All manipulations of *F. tularensis* strain Schu S4 and its derivatives were performed in a biosafety level-3 facility according to standard operating procedures approved by the Rocky Mountain Laboratories Institutional Biosafety Committee.

Construction of in-frame deletion mutants of Schu S4. In-frame deletion mutants of Schu S4 were generated using the SacB-assisted allelic replacement suicide vector pJC84, as described previously (46). Each deletion was designed to preserve the integrity of the downstream gene(s) (see Fig. 2) and to avoid any polar effect of the deletion. To engineer in-frame deletions of either *acpA* (the FTT0221 locus), *acpB* (the FTT0156 locus), or *acpC* (the FTT0620 locus), 5′ hemifragments, containing upstream regions and the start codon of each gene, and 3′ hemifragments, containing the last 6 codons of each gene and downstream regions, were generated by PCR amplification (Table 1). Both hemifragments were fused by overlap extension PCR, cloned into pCR2.1 TOPO (Invitrogen), and fully sequenced. The resulting fragments contained residual ORFs of the last 6 codons of *acpA*, *acpB*, and *acpC*, respectively, and were subcloned into pJC84 using the BamHI and SalI sites introduced by PCR primers (Table 1) to produce pJC84Δ*acpA*, pJC84Δ*acpB*, and pJC84Δ*acpC*, respectively.

For allelic replacement in the chromosome of Schu S4, electrocompetent bacteria were prepared and electroporated with recombinant pJC84 plasmid DNA as previously described (46). Kanamycin-resistant merodiploid colonies were tested for integration of the allelic replacement plasmid, using colony PCR with primers JC420 and JC427 (to amplify a 1.5-kb internal fragment of *sacB*) or primers JC589 and JC428 (to amplify a 900-bp fragment of the pJC84 backbone). Independent clones were then subjected to sucrose counterselection as previously described (46) in order to isolate clones that had undergone allelic replacement. The presence of the deleted allele and allelic replacement within the correct chromosomal locus were verified by PCR using primers JC704 and JC705 and primers JC706 and JC707, respectively, for the *acpA* deletion, primers JC736 and JC737 and primers JC738 and JC739, respectively, for the *acpB* deletion, primers JC728 and JC729 and primers JC730 and JC731, respectively, for the *acpC* deletion, and primers JC420 and JC427 for the loss of the *sacB* gene (Table 1). Independent clones carrying the correct in-frame deletion in either *acpA*, *acpB*, or *acpC* were isolated and used for further studies. Multiple deletion mutants were generated by repeating the allelic replacement procedure on single (Δ*acpA*) or double (Δ*acpAB*) deletion mutants.

Acid phosphatase assay. To measure acid phosphatase activity associated with either Schu S4 or its isogenic acid phosphatase mutants, strains were grown overnight at 37°C in MMH broth under shaking conditions, and cultures were normalized based on readings of optical density at 600 nm (OD_{600}). Bacteria were collected by centrifugation at 6,000 × *g* and 4°C for 10 min, and the pellets were washed twice with phosphate-buffered saline (PBS) and resuspended in 1/100 of the original culture volume in PBS. Cells were transferred to 2 ml Lysing Matrix B FastPrep tubes (Qbiogene, Carlsbad, CA) and were lysed through nine cycles of 45 s each at a speed of 6.5 m/s with a FastPrep 120 instrument (Qbiogene); tubes were placed on ice for 1.5 min between each cycle. Lysates were collected and centrifuged at 6,000 × *g* and 4°C for 10 min to remove any unbroken cells and cell debris. Total-protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA), and samples were normalized for protein content before acid phosphatase activity was measured by the release of *p*-nitrophenol (pNP) from *p*-nitrophenylphosphate (2) using an acid phosphatase assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Measurements were expressed as acid phosphatase activity units per milligram of total protein, where 1 U of specific activity is defined as that which yields 1 nM pNP per min at 37°C and pH 4.8. Results were expressed as percentages of the phosphatase activity measured for the wild-type strain.

Macrophage culture and infection. To generate murine bone marrow-derived macrophages (muBMMs), bone marrow cells were isolated from femurs of 6- to 10-week-old C57BL/6J female mice (Jackson Laboratories, Bar Harbor, ME), differentiated into macrophages as described previously (12), and replated in 24-well cell culture-treated plates at a density of 1×10^5 macrophages/well. Human monocyte-derived macrophages (MDMs) were generated from peripheral blood monocytes subjected to apheresis and enriched by density centrifugation using Ficoll-Paque (GE Healthcare) and by negative selection using the Dynabeads Untouched Human Monocytes kit (Invitrogen) according to the manufacturer's instructions. Mononuclear cells were seeded at a density of 4×10^5/well in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% nonessential amino acids (NEAA; Invitrogen), and 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF; PeproTech Inc., Rocky Hill, NJ). The medium was replenished on day 3 and day 6 of culture, and MDMs were used for infections on day 7. Human blood cells were collected from anonymous volunteers under a protocol reviewed and approved by the NIH Institutional Biosafety Committee. Immediately prior to infection of macrophages, a few colonies from a freshly streaked plate were resuspended in

TABLE 1. Primers used in this study

Primer function and name	Sequence (5′–3′)[a]	Nature of amplicon
pJC84 chromosomal detection		
JC420	CTAGCTAGCAGGAGACATGAACGATGAACATC	1.5-kb internal fragment of *sacB*
JC427	GGGACGTCGGATTCACCTTTATGTTGATAAG	1.5-kb internal fragment of *sacB*
JC428	GGGACGTCGATTAAGCATTGGTAACTGTCAGACC	900-bp fragment of the pJC84 backbone
JC589	ATCAGCTCACTCAAAGGCGG	900-bp fragment of the pJC84 backbone
Deletion of *acpA*		
JC700	TGGATCCTCTGTAACCACATTAGTATC	1,137 bp upstream and start codon of *acpA*
JC701	GTTTAATTTATCCACTACCATATGATACCTTTAGTTGT	1,137 bp upstream and start codon of *acpA*
JC702	TTTGTCGACTGCTAATACCGAGAGGTCAGCC	Last 6 codons of *acpA* and 1,006 bp downstream
JC703	GTAGTGGATAAATTAAACTAAA	Last 6 codons of *acpA* and 1,006 bp downstream
JC704	TCTCCTAACTAACTTATTTTTG	Δ*acpA*
JC705	GAAGAGGCAATAAGCGAAAATAC	Δ*acpA*
JC706	GTTCCAACTTATCGCTCCAAG	Δ*acpA* within the FTT0221 locus
JC707	GGAGTGGTTGCTGAATATGC	Δ*acpA* within the FTT0221 locus
Deletion of *acpB*		
JC732	TGGATCCGCTATGATTAGTAAATCTAGC	1,035 bp upstream and start codon of *acpB*
JC733	AATCTTGAGTTCTCACCCATAGAATTATTTTAGACCT	1,035 bp upstream and start codon of *acpB*
JC734	TTGGTCGACACATAACTTGTAGTATGAACT	Last 6 codons of *acpB* and 989 bp downstream
JC735	GGTGAGAACTCAAGATTTTAG	Last 6 codons of *acpB* and 989 bp downstream
JC736	GATAAATATCCAAGAGCTGAG	Δ*acpB*
JC737	CGATTACGATAAGTTGTGCTA	Δ*acpB*
JC738	GAGATCGGTACAGCTATTGCTT	Δ*acpB* within the FTT0156 locus
JC739	CAAGTAGCATAAATCATCGCG	Δ*acpB* within the FTT0156 locus
Deletion of *acpC*		
JC724	TGGATCCTCATCTCTGGAAGTCTATGA	1,254 bp upstream and start codon of *acpC*
JC725	GCCAGCTGCCATACATCATTGATGTTTCTAAGTTCTT	1,254 bp upstream and start codon of *acpC*
JC726	TTGGTCGACACTATCCTTCTTTAGTTGCT	Last 6 codons of *acpC* and 635 bp downstream
JC727	ATGTATGGCAGCTGGCAATAA	Last 6 codons of *acpC* and 635 bp downstream
JC728	ACATTCTCTGGCGTGGTGAG	Δ*acpC*
JC729	CCAGCATCCATAGCCGAATA	Δ*acpC*
JC730	TGGCTCTGGTAGATAATCA	Δ*acpC* within the FTT0620 locus
JC731	TTACTACTCAGGCGTTGAAAC	Δ*acpC* within the FTT0620 locus

[a] Underlined sequences denote either the BamHI (GGATCC) or the SalI (GTCGAC) restriction site used for cloning into pJC84.

MMH broth, and the OD_{600} was measured to estimate bacterial numbers. Bacteria were diluted to the appropriate multiplicity of infection (MOI) in either muBMM or MDM medium, and 0.5 ml of the bacterial suspension was added to chilled cells. Macrophage infections were then performed as described previously (12) at an applied MOI of 25 to 100, depending on the analysis performed.

Determination of intracellular bacterial growth. The intracellular growth of Schu S4 and its derivatives was monitored by determining the number of CFU recovered from lysed macrophages, as described previously (12). The number of viable intracellular bacteria per well was determined in triplicate for each time point.

Immunofluorescence microscopy. BMMs grown on 12-mm-diameter glass coverslips in 24-well plates were infected at an MOI of 25 and processed for immunofluorescence labeling as described previously (12). The primary antibodies used were mouse anti-*F. tularensis* lipopolysaccharide (United States Biological, Swampscott, MA), rat anti-mouse LAMP-1 (clone 1D4B, developed by J. T. August and obtained from the Developmental Studies Hybridoma Bank [a national resource developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242]). The secondary antibodies were Alexa Fluor 488-conjugated donkey anti-mouse and Alexa Fluor 568-conjugated donkey anti-rat antibodies (Invitrogen). To quantify the escape of *Francisella* from its initial phagosome, phagosomal integrity assays were performed as described previously (12). Samples were observed on a Carl Zeiss Axio Imager epifluorescence microscope equipped with a Plan-Apochromat 63× objective (numerical aperture, 1.4) for quantitative analysis or on a Carl Zeiss LSM 710 confocal laser scanning microscope for image acquisition. Confocal images of 1,024 by 1,024 pixels were acquired and assembled using Adobe Photoshop CS3.

Transmission electron microscopy. BMMs on 12-mm-diameter Aclar coverslips were infected at an MOI of 100 and processed as described previously (12). Sections were viewed in a Hitachi H7500 transmission electron microscope at 80 kV. Images were acquired with a Hamamatsu 2,000-by-2,000-pixel bottom-mount digital camera (Advanced Microscopy Techniques, Danvers, MA) and were assembled in Adobe Photoshop CS3.

Animal infections. Groups of 10 BALB/cJ mice, 6 to 8 weeks old (Jackson Laboratories), were infected with wild-type *F. tularensis* Schu S4 or the indicated mutant strains via the intranasal route for survival studies. Immediately prior to infection, a stock vial of bacteria was thawed and serially diluted in PBS to the appropriate bacterial density. Mice were anesthetized intraperitoneally with 100 μl of a solution containing 12.5 mg/ml ketamine plus 3.8 mg/ml xylazine. For intranasal infections, approximately 10 CFU in 25 μl of PBS was administered to the nares of each mouse. Actual doses were confirmed by plating the inoculum on MMH agar plates. Animals were monitored twice daily for signs of morbidity and were euthanized when moribund. All animal infections were performed at biosafety level 3 and were approved by the Rocky Mountain Laboratories Animal Care and Use Committee.

Nucleotide sequence accession numbers. The NCBI reference sequence (RefSeq) numbers of the *Francisella acp* and *hap* genes are as follows: for U112 *acpA*, YP_897755; for Schu S4 *acpA*, YP_169276; for FSC200 *acpA*, ZP_02275294; for U112 *acpB*, YP_899174; for Schu S4 *acpB*, YP_169222; for FSC200 *acpB*, ZP_02274312; for U112 *acpC*, YP_898702; for Schu S4 *acpC*, YP_169641; for FSC200 *acpC*, ZP_02275790 and ZP_02275789; for U112 *acpD*, YP_898327; for Schu S4 *acpD*, YP_169785 and YP_170416; for FSC200 *acpD*, ZP_02274752 and ZP_02275415; for U112 *hapA*, YP_898596; for Schu S4 *hapA*,

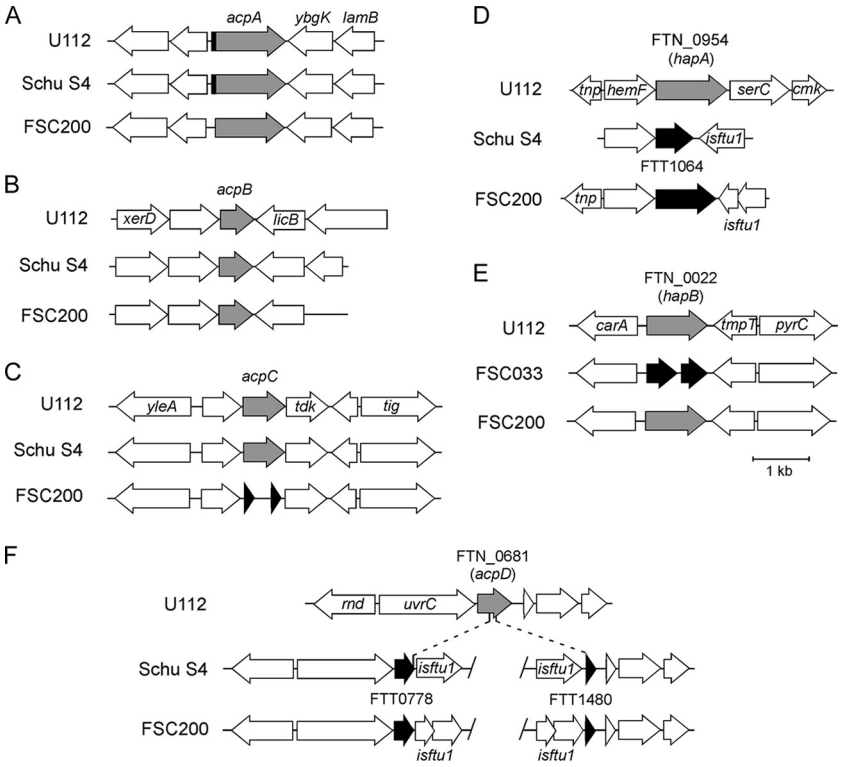

FIG. 1. Comparative genetic organizations of acid phosphatase-encoding loci in *F. novicida*, *F. tularensis* subsp. *tularensis*, and *F. tularensis* subsp. *holarctica*. Shown are the genetic organizations of the *acpA* (A), *acpB* (B), *acpC* (C), *hapA* (D), *hapB* (E), and *acpD* (F) loci in *F. novicida* U112, *F. tularensis* subsp. *tularensis* Schu S4 (A through D and F) or FSC033 (E), and *F. tularensis* subsp. *holarctica* FSC200. (A) The *acpA* gene (shaded arrow) is highly conserved between subspecies yet lacks the first 23 codons, including a putative secretion signal peptide (small filled rectangle), in *F. tularensis* subsp. *holarctica* strains. (B) The *acpB* gene (shaded arrow) is highly conserved between subspecies. (C) The *acpC* gene (shaded arrow) is conserved between the *F. novicida* and *F. tularensis* subsp. *tularensis* strains but is disrupted (filled arrows) in the *F. tularensis* subsp. *holarctica* strain. (D) The *hapA* gene (FTN_0954) (shaded arrow) is truncated (filled arrows) by IS*ftu1*-mediated chromosomal rearrangements in both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains. (E) The *hapB* gene (FTN_0022) (shaded arrow) is intact in *F. novicida* and *F. tularensis* subsp. *holarctica* strains but is a pseudogene (filled arrows) in the *F. tularensis* subsp. *tularensis* strain. (F) The *acpD* gene is intact (FTN_0681) in the *F. novicida* strain but is disrupted (filled arrows) by IS*ftu1*-mediated chromosomal rearrangements in both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains.

YP_170045; for FSC200 *hapA*, ZP_02275376; for U112 *hapB*, YP_897687; and for FSC200 *hapB*, ZP_02274253.

RESULTS

Degeneration of acid phosphatase-encoding genes in virulent strains. To evaluate the contribution of acid phosphatases to the virulence of Schu S4, we first examined the conservation of the genetic loci encoding such enzymes in the annotated genome sequences of *F. novicida* strain U112, *F. tularensis* subsp. *tularensis* strains Schu S4 and FSC033, and *F. tularensis* subsp. *holarctica* strain FSC200 by using the ERGO Genome Analysis Suite (Integrated Genomics, Chicago, IL) (34). Analysis of the U112 genome identified the acid phosphatase-encoding genes *acpA* (FTN_0090), *acpB* (FTN_1556), and *acpC* (FTN_1061) (Fig. 1), a PAP2 superfamily phosphoesterase-encoding gene named *acpD* (FTN_0681) (Fig. 1), and the two histidine acid phosphatase-encoding genes *hapA* (FTN_0954) and *hapB* (FTN_0022), the latter of which shares 41% amino acid identity with the *Legionella pneumophila* major acid phosphatase Map (16). The *acpA* locus was conserved between the U112 and Schu S4 genomes (Fig. 1A), with the deduced AcpA proteins sharing 98.1% identity (see Fig. S1 in the supplemental material), while the *acpA* loci of FSC200 (see Fig. S1) and two other *F. tularensis* subsp. *holarctica* strains (data not shown) encoded an AcpA protein missing the N-terminal 23 amino acid residues and therefore lacking a potential Sec-dependent signal peptide identified using SignalP, version 3.0 (5). The *acpB* locus was highly conserved between the U112, Schu S4, and FSC200 genomes, with 99% identity between the deduced amino acid sequences (Fig. 1B; see also Fig. S1 in the supplemental material), underscoring a potentially important function of this gene. Like that of *acpA*, the *acpC* locus was highly conserved between the U112 (FTN_1061) and Schu S4 (FTT0620) genomes, with 96.4% identity at the protein level, but was disrupted into two small ORFs in FSC200 (Fig. 1C; see also Fig. S1 in the supplemental material). A similar genetic degeneration was observed in two other *F. tularensis* subsp. *holarctica* genomes (data not shown), indicating that *acpC* is a pseudogene in type B strains. Compared to that in U112, the *hapA* gene in Schu S4 (FTT1064) was truncated through an IS*ftu1*-mediated chromosomal rearrangement (Fig. 1D; see also Fig. S1 in the supplemental material), generating, instead of a 402-amino-acid protein, a 196-amino-acid protein that lacks the C-terminal catalytic histidine (His309) residue typical

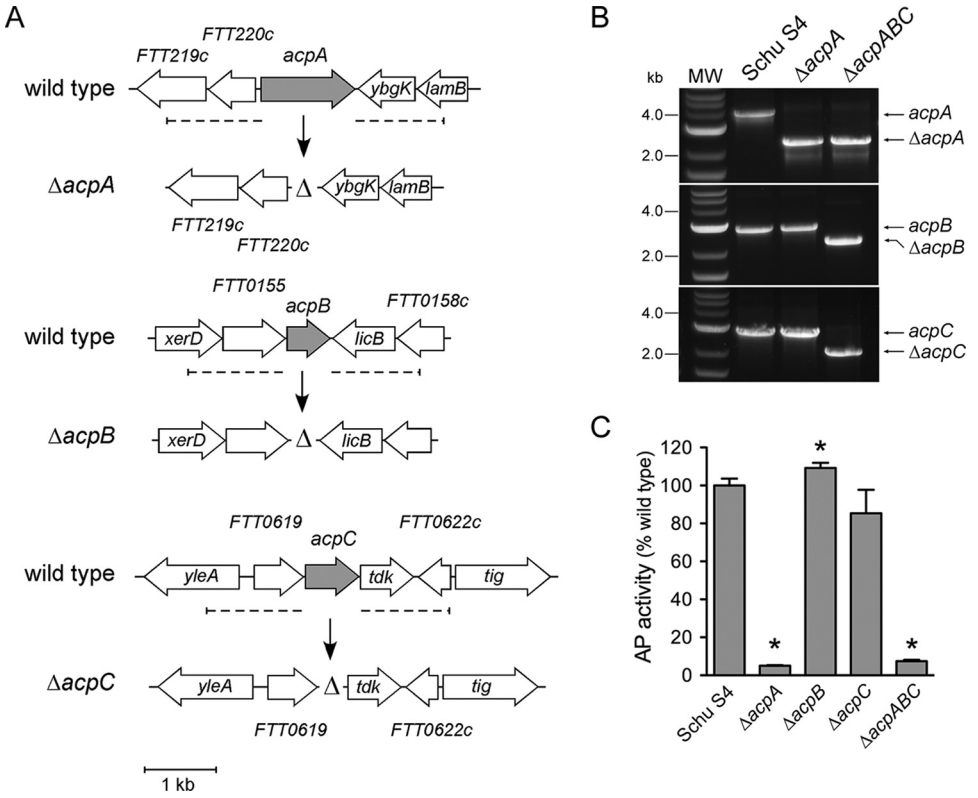

FIG. 2. Construction of Δ*acpA* and Δ*acpABC* Schu S4 mutants. (A) Schematic representation of the *acpA*, *acpB*, and *acpC* loci in the Schu S4 chromosome before and after allelic replacement using pJC84Δ*acpA*, pJC84Δ*acpB*, and pJC84Δ*acpC*, respectively (see Materials and Methods). Dashed lines indicate the chromosomal regions flanking the respective loci that were used for allelic replacement. The allelic replacement was designed to preserve the integrity of the *ybgK* gene, located immediately downstream of *acpA*; of the *licB* gene, located immediately downstream of *acpB*; and of the *tdk* gene, located immediately downstream of *acpC*. (B) PCR confirmation of *acpA* (top), *acpB* (center), and *acpC* (bottom) deletions from the correct Schu S4 chromosomal loci in the Δ*acpA* and Δ*acpABC* mutants. Both wild-type and deleted amplified regions are indicated. (C) Acid phosphatase (AP) activities in Schu S4 and *acp* mutants. Whole-cell lysates were generated from bacterial cultures and assayed for AP activity as described in Materials and Methods. Values are means ± standard deviations for three independent experiments. Asterisks indicate statistically significant differences ($P < 0.001$) from wild-type activity by a two-tailed Student *t* test.

of this family of histidine acid phosphatases (32, 33). Through similar rearrangements, *hapA* in FSC200 was also truncated to generate a 351-amino-acid residue protein. Hence, though present in virulent strains (29), *hapA* is likely a pseudogene. *hapB* (FTN_0022) was found to be intact in FSC200 but disrupted into two ORFs in the type B strain FSC022 (data not shown) and the type A strain FSC033 (Fig. 1E). Although *hapB* is not annotated in the Schu S4 genome, sequences with 100% identity with the FSC033 ORFs were found in the Schu S4 genome sequence, indicating that *hapB* is present yet disrupted in type A strains, but not absent as was previously reported (29). We found an additional, undescribed PAP2 superfamily acid phosphatase, named AcpD, encoded by the U112 locus FTN_0681 (Fig. 1F; see also Fig. S1 in the supplemental material), raising the possibility of additional acid phosphatase activity in *F. novicida*. However, *acpD* homologs in both Schu S4 and FSC200 were disrupted into two pseudogenes (FTT0778 and FTT1480 in Schu S4 [Fig. 1F]) by obvious IS*ftu1*-mediated chromosomal rearrangements, where the two gene remnants were located in distant regions of the Schu S4 or FSC200 chromosome (Fig. 1F). Hence, though intact in *F. novicida*, *acpD* is disrupted in virulent type A and type B strains of *Francisella*. Taken together, this analysis highlighted a lack of conservation of acid phosphatase-encoding genes between *F. novicida* and the virulent *F. tularensis* subspecies, where most of these genes have been converted into pseudogenes in at least one virulent subspecies through genome reduction events (10). Given the previously documented roles of AcpA, AcpB, and AcpC in the phagosomal escape and virulence of *F. novicida* (27, 29), we nonetheless examined the roles of all three of these genes in the intracellular trafficking and virulence of Schu S4.

Deletion of the *acpABC* genes in Schu S4 abolishes its acid phosphatase activity. To address the roles of AcpA, AcpB, and AcpC in the virulence of Schu S4, we generated either single in-frame, unmarked deletions of *acpA*, *acpB*, and *acpC* or combinations of those deletions (Fig. 2A). As shown in Fig. 2B for the Schu S4 Δ*acpA* and Schu S4 Δ*acpA* Δ*acpB* Δ*acpC* (referred to below as Δ*acpABC*) mutants, all deletions were confirmed by PCR amplification of the deleted gene(s) within the proper chromosomal locus. Compared to the parental strain, none of the mutants generated showed any growth defect in culture in MMH broth (data not shown), indicating that *acp* deletions do not affect the ability of Schu S4 to replicate in a synthetic growth medium. Deletion of *acpA* decreased the total acid phosphatase activity of Schu S4 by 95% (Fig. 2C),

consistent with previous reports on *F. novicida* (4, 27, 29), arguing that AcpA accounts for most acid phosphatase activities in *Francisella*. Deletion of *acpB* or *acpC* by itself did not dramatically affect acid phosphatase activity levels (Fig. 2C), suggesting that these proteins express only marginal, if any, acid phosphatase activities. In agreement with these results, the total acid phosphatase activity of the Δ*acpABC* triple deletion mutant was decreased to a level comparable to that of the Δ*acpA* mutant (Fig. 2C). All three genes were transcribed on MMH plates (46; also unpublished data), suggesting that the lack of detectable acid phosphatase activity associated with AcpB and AcpC is not due to a lack of expression. Taken together, these results demonstrate that the *acp* deletion mutants displayed significantly reduced acid phosphatase activity, mostly via deletion of *acpA*.

AcpABC are not required for the intracellular cycle of Schu S4. To determine whether the acid phosphatases AcpA, AcpB, and AcpC play a role in the intracellular pathogenesis of Schu S4, we first compared the intracellular growth of either the Schu S4 Δ*acpA* or the Schu S4 Δ*acpABC* mutant with that of the parental Schu S4 strain in either murine bone marrow-derived macrophages (muBMMs) or human blood monocyte-derived macrophages (MDMs). Over a time course of 12 h, which typically encompasses phagosomal escape and cytosolic replication (46), numbers of viable intracellular Δ*acpA* and Δ*acpABC* mutants were indistinguishable from numbers of viable intracellular Schu S4 (Fig. 3A and B), indicating that these deletions do not affect the ability of Schu S4 to proliferate within murine or human macrophages. Under these experimental conditions, both muBMMs and MDMs were capable of controlling an attenuated strain of *Francisella*, since a *fevR* deletion mutant (7, 46) was unable to survive intracellularly (Fig. 3).

To further examine the intracellular behavior of the *acp* mutants, we monitored their intracellular trafficking in muBMMs by using fluorescence confocal and transmission electron microscopy. At 1 h postinfection (p.i.), neither Schu S4 nor the Δ*acpA* or Δ*acpABC* mutant was surrounded by LAMP-1-positive membranes (Fig. 4A, B, and C), suggesting they all had disrupted their phagosomal membranes. Electron microscopy showed that, like Schu S4, neither the Δ*acpA* nor the Δ*acpABC* mutant was surrounded by phagosomal membranes at this time point, demonstrating efficient phagosomal membrane disruption at the ultrastructural level (Fig. 4A, B, and C). To quantitatively evaluate phagosomal escape, muBMMs infected with either Schu S4, Schu S4 Δ*acpA*, Schu S4 Δ*acpABC*, or the phagosomal escape-deficient strain Schu S4 Δ*fevR* (46) were subjected to a phagosomal integrity assay. While less than 20% of intracellular Δ*fevR* bacteria were cytosolic during the first 4 h p.i., consistent with our previous results (46), the percentages of cytosolic Δ*acpA* and Δ*acpABC* mutants paralleled those of wild-type Schu S4 (Fig. 4G), demonstrating that these mutations did not affect the efficiency or the kinetics of phagosomal disruption and bacterial release into the cytosol. At 8 h p.i., both the Δ*acpA* and Δ*acpABC* mutants showed clear patterns of cytosolic replication similar to those of Schu S4 (Fig. 4D, E, and F), confirming intracellular proliferation. Taken together, our results clearly show a normal intracellular cycle of acid phosphatase-deficient bacteria, ruling out a role for these acid phosphatases in the intracellular pathogenesis of Schu S4.

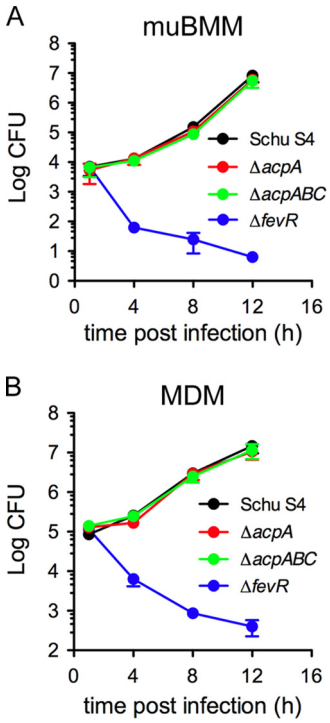

FIG. 3. Deletion of *acp* genes in Schu S4 does not affect intracellular growth in murine or human macrophages. muBMMs or MDMs were infected with either the Schu S4, Schu S4 Δ*acpA*, Schu S4 Δ*acpABC*, or Schu S4 Δ*fevR* strain, and viable intracellular bacteria were enumerated as CFU in a gentamicin protection assay. Growth curves of wild-type and mutant Schu S4 strains in muBMMs (A) and MDMs (B) are shown. In each case, results are representative of two independent experiments performed in triplicate.

AcpABC are not required for in vivo virulence of Schu S4. Although AcpA, AcpB, and AcpC are not required for Schu S4 intracellular pathogenesis, there remained the possibility of a role for these phosphatases in the overall virulence of *Francisella in vivo*. To address this question, we infected BALB/cJ mice intranasally with either Schu S4, Schu S4 Δ*acpA*, or the Schu S4 Δ*acpABC* triple mutant and monitored their survival. In this infection model, Schu S4 mutants either defective in phagosomal escape or impaired in cytosolic proliferation fail to cause any lethality (46). Regardless of the infecting strain, mice from all groups became moribund and had to be euthanized by day 4 p.i. (Fig. 5), indicating that deletion of either *acpA* alone or *acpA*, *acpB*, and *acpC* together does not affect the virulence of Schu S4 *in vivo*.

DISCUSSION

Despite the discovery of the FPI and its documented role in the phagosomal escape of *Francisella* species, very little is known about other molecular determinants of the intracellular pathogenesis of this bacterium. Recently, Mohapatra et al. have examined the role of the acid phosphatases AcpA, AcpB, AcpC, and Hap in pathogenesis and concluded that these proteins are required for the phagosomal escape, and hence for

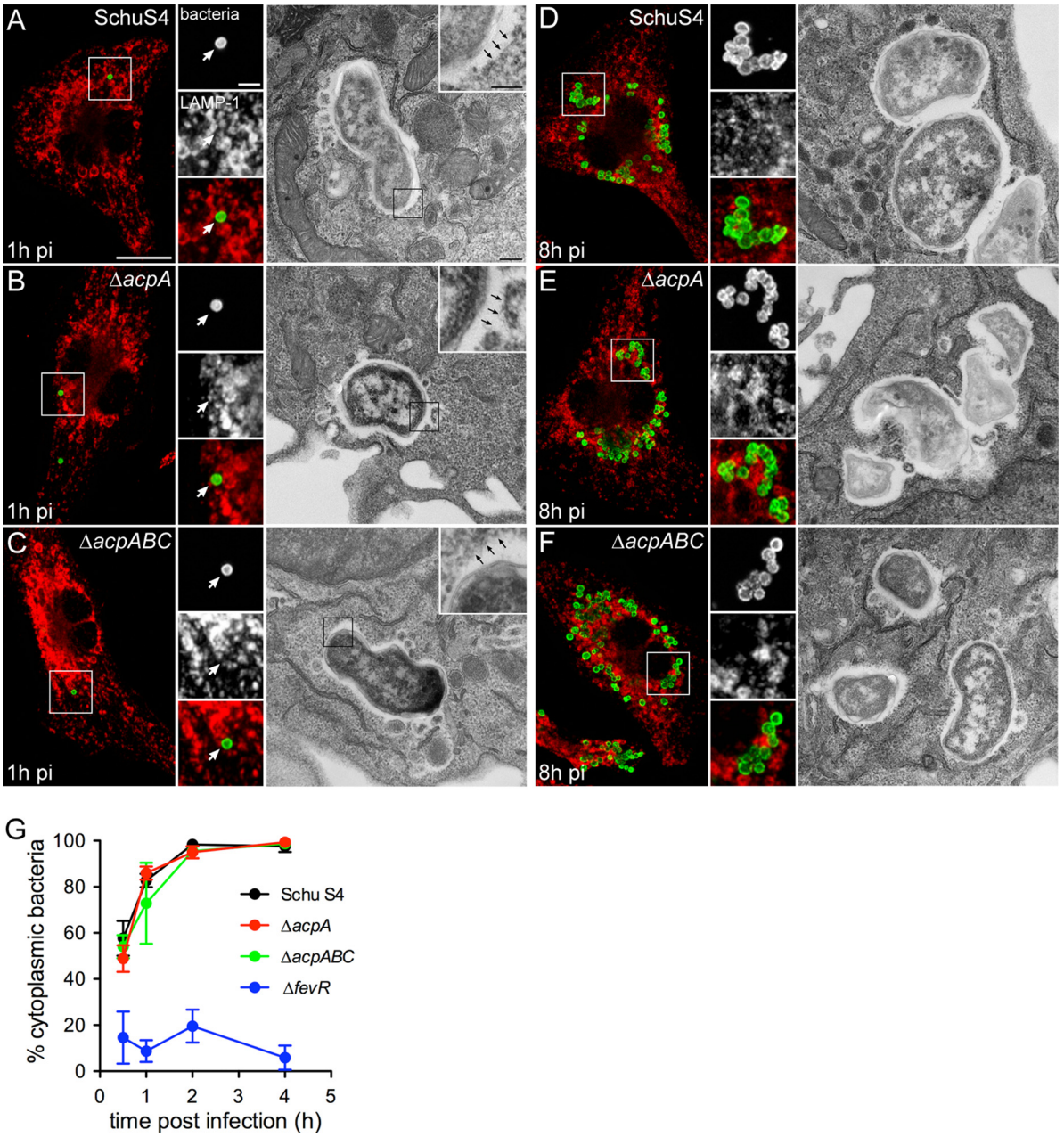

FIG. 4. Intracellular trafficking of Schu S4 is not affected by deletion of the *acp* genes. muBMMs were infected with strain Schu S4, Schu S4 Δ*acpA*, or Schu S4 Δ*acpABC* and were processed for either immunofluorescence or transmission electron microscopy. (A to F) Representative confocal fluorescence or electron micrographs of muBMMs at 1 h (A to C) or 8 h (D to F) after infection with either Schu S4 (A and D), Schu S4 Δ*acpA* (B and E), or Schu S4 Δ*acpABC* (C and F). Bacteria appear green; LAMP-1 appears red. White arrows on the confocal micrograph insets indicate bacteria that are not surrounded by LAMP-1-positive membranes. Black arrows on the electron micrograph insets indicate a lack of phagosomal membranes. Bars, 10 and 2 μm for confocal microscopy panels and insets, respectively; 0.5 and 0.2 μm for electron microscopy panels and insets, respectively. (G) Phagosomal escape kinetics of strains Schu S4, Schu S4 Δ*acpA*, Schu S4 Δ*acpABC*, and Schu S4 Δ*fevR* in muBMMs. Macrophages were infected with individual strains, and were processed for a phagosomal integrity assay, as described in Materials and Methods. Values are means ± standard deviations for three independent experiments.

the intracellular growth, of *F. novicida* (27, 29). This contradicted a previous report of a lack of a role for AcpA in intracellular growth and virulence in the same organism (4). In our efforts to identify pathogenic determinants of the prototypical type A strain Schu S4, we sought to examine the roles played by acid phosphatase-encoding genes. Because phagosomal escape is crucial to the pathogenesis of all *Francisella* species, we postulated that bacterial factors required for this process are highly conserved, including acid phosphatase-encoding genes, based on their role in phagosomal disruption in *F. novicida* (27, 29). Here, however, we show poor conservation of these bacterial determinants between *F. novicida* and the virulent *F.*

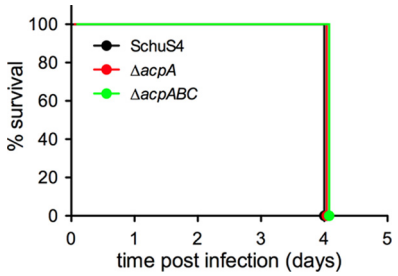

FIG. 5. Deletion of the *acp* genes does not affect the virulence of Schu S4. Survival curves of BALB/cJ mice infected intranasally with either Schu S4 or its isogenic Δ*acpA* or Δ*acpABC* mutant are shown. Inocula were 11 (Schu S4), 15 (Schu S4 Δ*acpA*), and 11 (Schu S4 Δ*acpABC*) CFU.

tularensis subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains. While *acpD*, *hapA*, and *hapB* are pseudogenes in *F. tularensis* subsp. *tularensis* strains, *acpA* is truncated at its 5′ end, and *acpC*, *acpD*, and *hapA* are pseudogenes, in *F. tularensis* subsp. *holarctica* strains. Degeneration of these genes in the virulent subspecies argues for a lack of selective pressure in the context of *Francisella* pathogenesis and hence for a nonessential role, if any. To confirm that such disrupted genes do not express any residual virulence-related functions and to exclude any possibility of reconstitution of a functional enzyme through the expression of the two pseudogene fragments (Fig. 1F; see also Fig. S1 in the supplemental material), we deleted FTT0778 (which is homologous to the 5′ portion of *F. novicida acpD*) in either Schu S4 or Schu S4 Δ*acpABC*. Regardless of the strain background, we observed no decrease in acid phosphatase activity and no defect in the infection cycle or virulence in mice (data not shown). These results confirm the nonfunctionality of *acpD* remnants in virulent strains and argue against a contribution of genes that have been subject to genome reduction to *Francisella* virulence.

We nonetheless deleted in Schu S4 the acid phosphatase-encoding genes that are most conserved (*acpA*, *acpB*, *acpC*) between *F. novicida* and most virulent *F. tularensis* subspecies, but the resulting mutants showed no defect in intracellular pathogenesis. Additionally, the ability of these mutants to cause lethality in mice was not impaired. Although it remains possible that *acpA*, *acpB*, and *acpC*, or one of these genes, play minor roles in the disease process that were not revealed in our intranasal infection model, our results indicate that these genes are not major determinants of Schu S4 virulence. Given its prominent acid phosphatase activity (Fig. 2C) (29), its demonstrated phospholipase C activity (37), and its putative secretion signal, AcpA appeared a likely candidate for a role in phagosomal membrane disruption, yet the phagosomal escape of the Δ*acpA* mutant of Schu S4 was unaffected. Additionally, the deletion at the 5′ end of *acpA* in *F. tularensis* subsp. *holarctica* strains has removed any putative secretion signal (see Fig. S1 in the supplemental material), yet this does not prevent type B strains from efficiently disrupting their early phagosome (11), excluding a yet to be demonstrated secretion of AcpA as a requirement for phagosomal degradation. Neither AcpB nor AcpC contributed in a significant manner to the total acid phosphatase activity of Schu S4, nor were they involved in phagosomal escape or virulence. While AcpA has been extensively characterized as an enzyme (4, 17, 18, 37), the classification of AcpB and AcpC as HAD family acid phosphatases is based on Pfam domain analyses, and their actual molecular functions remain to be evaluated.

Taken together, our results rule out a role of acid phosphatases in the phagosomal escape and overall pathogenesis of highly virulent *F. tularensis* subspecies. These findings are therefore consistent with the study of *F. novicida* AcpA by Baron et al. (4) but contrast with the conclusions of Mohapatra et al. (27, 29). Although one could invoke differential requirements for bacterial virulence factors between murine macrophages (our main model in this study) and human monocytes (used in the study by Mohapatra et al.) to explain these different conclusions, we did not uncover any defect of the Schu S4 *acp* mutants in primary human macrophages either, ruling out this possibility. While additional studies are needed to reconcile previous reports on the roles of acid phosphatases in *F. novicida*, our findings that Acp proteins do not play a role in the virulence of type A strains call for a reconsideration of the generalized conclusion that acid phosphatases are required for the phagosomal escape of *Francisella*. More generally, the assumption that findings generated using *F. novicida* hold true for the more virulent *F. tularensis* subspecies should be viewed with caution and confirmed experimentally.

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REFERENCES

1. **Aragon, V., S. Kurtz, and N. P. Cianciotto.** 2001. Legionella pneumophila major acid phosphatase and its role in intracellular infection. Infect. Immun. **69:**177–185.
2. **Aragon, V., S. Kurtz, A. Flieger, B. Neumeister, and N. P. Cianciotto.** 2000. Secreted enzymatic activities of wild-type and pilD-deficient Legionella pneumophila. Infect. Immun. **68:**1855–1863.
3. **Baca, O. G., M. J. Roman, R. H. Glew, R. F. Christner, J. E. Buhler, and A. S. Aragon.** 1993. Acid phosphatase activity in Coxiella burnetii: a possible virulence factor. Infect. Immun. **61:**4232–4239.
4. **Baron, G. S., T. J. Reilly, and F. E. Nano.** 1999. The respiratory burst-inhibiting acid phosphatase AcpA is not essential for the intramacrophage growth or virulence of Francisella novicida. FEMS Microbiol. Lett. **176:**85–90.
5. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:**783–795.
6. **Bönquist, L., H. Lindgren, I. Golovliov, T. Guina, and A. Sjostedt.** 2008. MglA and Igl proteins contribute to the modulation of Francisella tularensis live vaccine strain-containing phagosomes in murine macrophages. Infect. Immun. **76:**3502–3510.
7. **Brotcke, A., and D. M. Monack.** 2008. Identification of fevR, a novel regulator of virulence gene expression in Francisella novicida. Infect. Immun. **76:**3473–3480.
8. **Brotcke, A., D. S. Weiss, C. C. Kim, P. Chain, S. Malfatti, E. Garcia, and D. M. Monack.** 2006. Identification of MglA-regulated genes reveals novel virulence factors in Francisella tularensis. Infect. Immun. **74:**6642–6655.
9. **Burtnick, M., A. Bolton, P. Brett, D. Watanabe, and D. Woods.** 2001. Identification of the acid phosphatase (acpA) gene homologues in pathogenic and non-pathogenic Burkholderia spp. facilitates TnphoA mutagenesis. Microbiology **147:**111–120.
10. **Champion, M. D., Q. Zeng, E. B. Nix, F. E. Nano, P. Keim, C. D. Kodira, M. Borowsky, S. Young, M. Koehrsen, R. Engels, M. Pearson, C. Howarth, L. Larson, J. White, L. Alvarado, M. Forsman, S. W. Bearden, A. Sjostedt, R. Titball, S. L. Michell, B. Birren, and J. Galagan.** 2009. Comparative genomic characterization of Francisella tularensis strains belonging to low and high virulence subspecies. PLoS Pathog. **5:**e1000459.
11. **Checroun, C., T. D. Wehrly, E. R. Fischer, S. F. Hayes, and J. Celli.** 2006. Autophagy-mediated reentry of Francisella tularensis into the endocytic compartment after cytoplasmic replication. Proc. Natl. Acad. Sci. U. S. A. **103:**14578–14583.

12. **Chong, A., T. D. Wehrly, V. Nair, E. R. Fischer, J. R. Barker, K. E. Klose, and J. Celli.** 2008. The early phagosomal stage of Francisella tularensis determines optimal phagosomal escape and Francisella pathogenicity Island protein expression. Infect. Immun. **76:**5488–5499.
13. **Clemens, D. L., B. Y. Lee, and M. A. Horwitz.** 2009. Francisella tularensis phagosomal escape does not require acidification of the phagosome. Infect. Immun. **77:**1757–1773.
14. **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.
15. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin. Microbiol. Rev. **15:**631–646.
16. **Felts, R. L., T. J. Reilly, M. J. Calcutt, and J. J. Tanner.** 2006. Crystallization of a newly discovered histidine acid phosphatase from Francisella tularensis. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. **62:**32–35.
17. **Felts, R. L., T. J. Reilly, and J. J. Tanner.** 2005. Crystallization of AcpA, a respiratory burst-inhibiting acid phosphatase from Francisella tularensis. Biochim. Biophys. Acta **1752:**107–110.
18. **Felts, R. L., T. J. Reilly, and J. J. Tanner.** 2006. Structure of Francisella tularensis AcpA: prototype of a unique superfamily of acid phosphatases and phospholipases C. J. Biol. Chem. **281:**30289–30298.
19. **Gallagher, L. A., E. Ramage, M. A. Jacobs, R. Kaul, M. Brittnacher, and C. Manoil.** 2007. A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate. Proc. Natl. Acad. Sci. U. S. A. **104:**1009–1014.
20. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt.** 2003. An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. Infect. Immun. **71:**5940–5950.
21. **Hall, J. D., M. D. Woolard, B. M. Gunn, R. R. Craven, S. Taft-Benz, J. A. Frelinger, and T. H. Kawula.** 2008. Infected-host-cell repertoire and cellular response in the lung following inhalation of Francisella tularensis Schu S4, LVS, or U112. Infect. Immun. **76:**5843–5852.
22. **Jungnitz, H., N. P. West, M. J. Walker, G. S. Chhatwal, and C. A. Guzman.** 1998. A second two-component regulatory system of Bordetella bronchiseptica required for bacterial resistance to oxidative stress, production of acid phosphatase, and in vivo persistence. Infect. Immun. **66:**4640–4650.
23. **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 intraamoebae and intramacrophage survival. Proc. Natl. Acad. Sci. U. S. A. **101:**4246–4249.
24. **Lindgren, H., I. Golovliov, V. Baranov, R. K. Ernst, M. Telepnev, and A. Sjostedt.** 2004. Factors affecting the escape of Francisella tularensis from the phagolysosome. J. Med. Microbiol. **53:**953–958.
25. **Ludu, J. S., O. M. de Bruin, B. N. Duplantis, C. L. Schmerk, A. Y. Chou, K. L. Elkins, and F. E. Nano.** 2008. The Francisella pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system. J. Bacteriol. **190:**4584–4595.
26. **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.
27. **Mohapatra, N. P., A. Balagopal, S. Soni, L. S. Schlesinger, and J. S. Gunn.** 2007. AcpA is a Francisella acid phosphatase that affects intramacrophage survival and virulence. Infect. Immun. **75:**390–396.
28. **Mohapatra, N. P., S. Soni, B. L. Bell, R. Warren, R. K. Ernst, A. Muszynski, R. W. Carlson, and J. S. Gunn.** 2007. Identification of an orphan response regulator required for Francisella virulence and transcription of pathogenicity island genes. Infect. Immun. **75:**3305–3314.
29. **Mohapatra, N. P., S. Soni, T. J. Reilly, J. Liu, K. E. Klose, and J. S. Gunn.** 2008. The combined deletion of four Francisella acid phosphatases attenuates virulence and macrophage vacuolar escape. Infect. Immun. **76:**3690–3699.
30. **Mougous, J. D., M. E. Cuff, S. Raunser, A. Shen, M. Zhou, C. A. Gifford, A. L. Goodman, G. Joachimiak, C. L. Ordonez, S. Lory, T. Walz, A. Joachimiak, and J. J. Mekalanos.** 2006. A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus. Science (New York) **312:**1526–1530.
31. **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. 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.
32. **Ostanin, K., E. H. Harms, P. E. Stevis, R. Kuciel, M. M. Zhou, and R. L. Van Etten.** 1992. Overexpression, site-directed mutagenesis, and mechanism of Escherichia coli acid phosphatase. J. Biol. Chem. **267:**22830–22836.
33. **Ostanin, K., A. Saeed, and R. L. Van Etten.** 1994. Heterologous expression of human prostatic acid phosphatase and site-directed mutagenesis of the enzyme active site. J. Biol. Chem. **269:**8971–8978.
34. **Overbeek, R., N. Larsen, T. Walunas, M. D'Souza, G. Pusch, E. Selkov, Jr., K. Liolios, V. Joukov, D. Kaznadzey, I. Anderson, A. Bhattacharyya, H. Burd, W. Gardner, P. Hanke, V. Kapatral, N. Mikhailova, O. Vasieva, A. Osterman, V. Vonstein, M. Fonstein, N. Ivanova, and N. Kyrpides.** 2003. The ERGO genome analysis and discovery system. Nucleic Acids Res. **31:**164–171.
35. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in Francisella tularensis. Nat. Rev. Microbiol. **2:**967–978.
36. **Pukatzki, S., A. T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W. C. Nelson, J. F. Heidelberg, and J. J. Mekalanos.** 2006. Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system. Proc. Natl. Acad. Sci. U. S. A. **103:**1528–1533.
37. **Reilly, T. J., G. S. Baron, F. E. Nano, and M. S. Kuhlenschmidt.** 1996. Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from Francisella tularensis. J. Biol. Chem. **271:**10973–10983.
38. **Remaley, A. T., R. H. Glew, D. B. Kuhns, R. E. Basford, A. S. Waggoner, L. A. Ernst, and M. Pope.** 1985. Leishmania donovani: surface membrane acid phosphatase blocks neutrophil oxidative metabolite production. Exp. Parasitol. **60:**331–341.
39. **Saha, A. K., J. N. Dowling, K. L. LaMarco, S. Das, A. T. Remaley, N. Olomu, M. T. Pope, and R. H. Glew.** 1985. Properties of an acid phosphatase from Legionella micdadei which blocks superoxide anion production by human neutrophils. Arch. Biochem. Biophys. **243:**150–160.
40. **Saleh, M. T., and J. T. Belisle.** 2000. Secretion of an acid phosphatase (SapM) by Mycobacterium tuberculosis that is similar to eukaryotic acid phosphatases. J. Bacteriol. **182:**6850–6853.
41. **Santic, M., R. Asare, I. Skrobonja, S. Jones, and Y. Abu Kwaik.** 2008. Acquisition of the vATPase proton pump and phagosome acidification is essential for escape of Francisella tularensis into the macrophage cytosol. Infect. Immun. **76:**2671–2677.
42. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. Modulation of biogenesis of the Francisella tularensis subsp. novicida-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-gamma. Cell. Microbiol. **7:**957–967.
43. **Santic, M., M. Molmeret, K. E. Klose, S. Jones, and Y. A. Kwaik.** 2005. The Francisella tularensis pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell. Microbiol. **7:**969–979.
44. **Schmerk, C. L., B. N. Duplantis, P. L. Howard, and F. E. Nano.** 2009. A Francisella novicida pdpA mutant exhibits limited intracellular replication and remains associated with the lysosomal marker LAMP-1. Microbiology **155:**1498–1504.
45. **Schmerk, C. L., B. N. Duplantis, D. Wang, R. D. Burke, A. Y. Chou, K. L. Elkins, J. S. Ludu, and F. E. Nano.** 2009. Characterization of the pathogenicity island protein PdpA and its role in the virulence of Francisella novicida. Microbiology **155:**1489–1497.
46. **Wehrly, T. D., A. Chong, K. Virtaneva, D. E. Sturdevant, R. Child, J. A. Edwards, D. Brouwer, V. Nair, E. R. Fischer, L. Wicke, A. J. Curda, J. J. Kupko III, C. Martens, D. D. Crane, C. M. Bosio, S. F. Porcella, and J. Celli.** 2009. Intracellular biology and virulence determinants of Francisella tularensis revealed by transcriptional profiling inside macrophages. Cell. Microbiol. **11:**1128–1150.
47. **Weiss, D. S., A. Brotcke, T. Henry, J. J. Margolis, K. Chan, and D. M. Monack.** 2007. In vivo negative selection screen identifies genes required for Francisella virulence. Proc. Natl. Acad. Sci. U. S. A. **104:**6037–6042.

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