IAI Accepts, published online ahead of print on 4 October 2010
Infect. Immun. doi:10.1128/IAI.00544-10
Copyright © 2010, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

The effects of the putative transcriptional regulator IclR on *Francisella tularensis* pathogenesis.

Brittany L. Mortensen, James R. Fuller, Sharon Taft-Benz, Todd M. Kijek, Cheryl N. Miller, Max T.H. Huang, and Thomas H. Kawula*

Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Running title: *Francisella tularensis* genes regulated by IclR

*Corresponding author

Department of Microbiology and Immunology
CB#7290, 804 MEJB
University of North Carolina
Chapel Hill, NC 27599-7290
Phone: 919.966.9699
FAX: 919.962.8200
Email: kawula@med.unc.edu

IAI Accepts published online ahead of print

Abstract

Francisella tularensis is a highly virulent Gram-negative bacterium and is the etiological agent of the disease tularemia. IclR, a presumed transcriptional regulator, is required for full virulence of the animal pathogen, *F. tularensis* subspecies *novicida* U112 (53). In this study, we investigated the contribution of IclR to the intracellular growth, virulence and gene regulation of human pathogenic *F. tularensis* subspecies. Deletion of *iclR* from the Live Vaccine and SchuS4 strains of *F. tularensis* subspecies *holarctica* and *tularensis,* respectively, did not affect their ability to replicate within macrophages or epithelial cells. In contrast to *F. tularensis* subspecies *novicida iclR* mutants, LVS and SchuS4 Δ*iclR* strains were equally virulent as their wild-type parental strains in intranasal inoculation mouse models of tularemia. Furthermore, wild-type LVS and LVSΔ*iclR* were equally cytotoxic and induced equivalent levels of IL-1β expression by infected bone marrow-derived macrophages. Microarray analysis revealed that the relative expression of a limited number of genes differed significantly between LVS wild-type and Δ*iclR* strains. Interestingly, many of the identified genes were disrupted in LVS and SchuS4 but not in their corresponding *novicida* U112 homologs. Thus, in spite of the impact of *iclR* deletion on gene expression, and in contrast to the effects of *iclR* deletion on *F. tularensis* subsp. *novicida* virulence, IclR does not contribute significantly to the virulence or pathogenesis of *F. tularensis* LVS or SchuS4.

Introduction

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

43 *Francisella tularensis* is a Gram-negative bacterium and the etiological agent of tularemia or
44 "rabbit fever". While zoonotic hosts include small mammals such as rabbits and voles, *F.*
45 *tularensis* is also found in ticks, mosquitoes, and flies, and can replicate within amoebae as well
46 (29). Human infection with *F. tularensis* can occur by several routes including bites by
47 arthropod vectors (4, 5, 34), contact with contaminated tissues, ingestion of contaminated food or
48 water (28, 43), or inhalation of aerosolized bacteria (18, 48). *F. tularensis* is considered a Select
49 Agent by the Centers for Disease Control due to its low infectious dose (as few as 10 organisms)
50 via the pulmonary route and its potential as a biological threat agent (15, 46).

51

52 There are two *F. tularensis* subspecies most commonly associated with disease in humans: *F.*
53 *tularensis* subspecies *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B). The
54 Live Vaccine Strain (LVS) of subsp. *holarctica* is a useful model for studying the virulent *F.*
55 *tularensis* subspecies, because it causes disease in mice, is attenuated in humans (19), and shares
56 genomic and proteomic similarity with *F. tularensis* subsp. *holarctica* and *tularensis* (51). *F.*
57 *tularensis* subsp. *novicida,* which does not cause disease in healthy humans, has significant
58 similarity with subsp. *holarctica* and *tularensis* and is also used as model organism for studying
59 *F. tularensis* pathogenesis. Although there are reports of subsp. *novicida* causing disease, these
60 cases are commonly associated with immunocompromised individuals (2, 9, 24, 32). However,
61 subsp. *novicida* does cause a severe disease in *in vivo* mouse models (40).

62

63 *Francisella* is known to predominately infect and replicate within macrophages but also infects
64 and replicates within neutrophils (37), dendritic cells (3) and Type II alveolar epithelial cells
65 (23). After phagocytosis, *F. tularensis* escapes the phagosome and replicates within the

IAI Accepts published online ahead of print

cytoplasm of host cells (1, 10). Numerous *in vitro* and *in vivo* screens have identified virulence factors required for this intracellular life cycle (13, 14, 27, 30, 35, 41, 47, 49, 53); however, many of the identified virulence factors have little or no similarity to known proteins of other bacteria and their functions remain, for the most part, unknown.

Weiss et al. recently identified a locus (FTN_0720) in *F. tularensis* subsp. *novicida* U112 that is important for virulence in mice as determined by an *in vivo* competition assay between a FTN_0720 deletion mutant and wild-type U112 (53). FTN_0720 encodes a protein with homology to the IclR family of transcriptional regulators. IclR family members activate and repress genes in a wide range of bacteria including genes involved in sporulation, metabolism, drug-efflux pumps and organic solvent tolerance, and phytopathogenicity (39). Given the close genetic relationship among the *F. tularensis* subspecies, the phenotype of the subsp. *novicida* *iclR* deletion strain suggests that IclR may be involved in the pathogenicity of the *holarctica* and *tularensis* subspecies. We investigated the contribution of IclR homologs in the pathogenicity of subsp. *holarctica* and *tularensis* by evaluating the role of IclR in gene expression, host cell interactions and virulence of *F. tularensis* subsp. *holarctica* LVS (FTL_1364) and subsp. *tularensis* SchuS4 (FTT_0748) strains.

Materials and Methods

Bacterial strains. *F. tularensis* subsp. *holarctica* LVS was obtained from the CDC, Atlanta, GA. *F. tularensis* subsp. *tularensis* SchuS4 was obtained from BEI Resources. *F. tularensis* subsp.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

novicida U112 was obtained from the American Type Culture Collection (ATCC). An *iclR* transposon mutant was one of two mutants from the transposon mutant library (21) and was received as a gift from Colin Manoil. All strains were maintained on chocolate agar supplemented with 1% IsoVitaleX (Becton-Dickson), brain heart infusion (BHI) broth supplemented with 1% IsoVitaleX or Chamberlain's defined medium (CDM) (6). *Escherichia coli* TOP10 (Invitrogen) were used for cloning purposes. *E. coli* was propagated in Luria broth supplemented with hygromycin at 200 µg/ml or kanamycin at 20 µg/ml as necessary for antibiotic selection. All cultures were grown at 37°C.

Cell Culture. J774A.1 (ATCC TIB-67) cells are a macrophage-like cell line derived from mouse sarcoma reticulum cells and were cultured in Dulbecco's minimal essential medium with 4.5 g/L glucose, 10% fetal bovine serum, and 2 mM $_{L}$-glutamine. TC-1 (ATCC CRL-2785) cells are a tumor cell line derived from mouse primary lung epithelial cells and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM $_{L}$-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES and 0.1 mM nonessential amino acids. Bone marrow-derived macrophages were generated by flushing bone marrow cells from C57BL/6 mouse femurs and recovered cells were incubated for 6 days on 15 cm^2 non-tissue culture-treated dishes in L929 cell-conditioned DMEM. Nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and bone marrow-derived macrophages were recovered from the dish using 1 mM EDTA in PBS.

Molecular techniques and allelic exchange. For both LVS and SchuS4 the *iclR* deletion was generated by splice overlap extension (SOE) PCR using primers designed to amplify the 5' and

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

111 3' regions of the *iclR* locus, which were then annealed to their complementary, homologous
112 genomic DNA tags (20, 25). The subsequent deletion left only the first six amino acids and the
113 stop codon of *iclR*. Each construct was cloned into the pCR-Blunt II TOPO vector (Invitrogen),
114 verified by DNA sequence analysis, and subsequently cloned into pMP590 (*sacB* Kan^r) using
115 *BamHI* and *NotI* restriction sites (20, 33). For allelic exchange, plasmids were electroporated into
116 LVS or SchuS4 and integrants were selected on chocolate agar containing kanamycin (10
117 µg/ml). Kan^r strains were grown overnight and plated on 10% sucrose for counterselection (loss
118 of plasmid) (20, 33). Both the LVS and SchuS4 *iclR* deletion strains were confirmed for loss of
119 *iclR* by PCR. For complementation of the *iclR* deletion in LVS, *iclR* and its predicted promoter
120 were PCR-amplified and subcloned into the pCR-Blunt II TOPO vector (Invitrogen). After a
121 *MluI/EcoRV* restriction digest, the construct was ligated into the pMP633 low-copy *Francisella*
122 shuttle vector (20) and electroporated into LVSΔ*iclR*. Complementation was determined by
123 detection of *iclR* in the complementation strain via PCR as well as demonstration of increased
124 *iclR* transcript levels via microarray analysis (data not shown).

125

126 **Gentamicin protection assays.** Gentamicin protection assays were performed as described (20,
127 23). Briefly, J774A.1 murine macrophages, TC-1 murine lung epithelial cells, or bone marrow-
128 derived macrophages were infected with LVS or SchuS4 at an MOI 100. Cells were incubated
129 with the bacterial inoculum for 2 hr (J774A.1 and bone marrow-derived macrophages) or 4 hr
130 (TC-1) and then incubated with media containing 25 µg/ml gentamicin for an additional 2 hr to
131 kill extracellular bacteria. At time points of 4 hr (or 6 hr for TC-1) and 24 hr, medium was
132 removed, cells were washed with PBS and then scraped from the dish, and the bacteria serially
133 diluted and plated to determine the number of viable bacteria.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

134

135 **Mouse infections.** 6- to 8-week-old C57BL/6 mice were anesthetized intraperitoneally (i.p) with
136 avertin and then inoculated intranasally (i.n.) with bacteria suspended in 50 µl PBS by
137 application to the nares of each mouse or inoculated intradermally (i.d.) by injection into the tail
138 using the same volume. Concentrations of LVS and U112 were determined by klett and
139 concentrations of SchuS4 by spectrophotometer (OD_{600}), and inocula were serially diluted and
140 plated on chocolate agar to confirm the CFU administered. At the designated time points, mice
141 were euthanized and the lungs, liver and spleen of each mouse were removed and homogenized.
142 Serial dilutions of the homogenates were plated on chocolate agar to enumerate the bacterial
143 organ burdens. Statistical significance between strains in each organ and at each time point was
144 determined by the Mann-Whitney nonparametric test using GraphPad Prism v.5 software. All
145 animal experiments were performed according to the animal care and use guidelines as
146 established by IACUC-approved protocols.

147

148 **IL-1β ELISAs and cytotoxicity assays.** Bone marrow-derived macrophages were seeded in 12-
149 well dishes at $1x10^6$ cells per well, and infected with bacteria at an MOI 500 in a final volume of
150 1 ml medium per well and incubated at 37°C. After 24 hr, the supernatants from each well were
151 collected, centrifuged to pellet cellular debris, and stored at -20°C. The IL-1β ELISA was
152 performed using the BD OptEIA mouse IL-1β ELISA kit (BD Biosciences) according to the
153 manufacturer's protocol. The OD_{450} was read using a TECAN Infinite M200 and analyzed using
154 Magellan v6 software. Cytotoxicity assays were performed using the ToxiLight® BioAssay kit
155 (Lonza) following the manufacturer's protocol for cytokine detection from supernatants, and the
156 luminescence was read using a TECAN Infinite M200 and analyzed using Magellan v6 software.

IAI Accepts published online ahead of print

Statistical significance between each strain was determined by the student's t-test using GraphPad Prism v.5 software.

Microarrays. RNA was obtained using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's protocol. Briefly, bacteria were grown to early mid-log phase in CDM and pelleted. Cells were disrupted by suspension in Trizol and vortexing with 0.1 mm glass beads. Purified RNA was recovered by chloroform extraction followed by treatment with DNase I to remove DNA. Microarray analysis was performed following the guidelines provided by the Venter Institute for Genomic Research (SOP#M007, M008). Briefly, aminoallyl labeled cDNA was generated from 2 µg total RNA using SuperScript III reverse transcriptase (Invitrogen), random hexamers, and dNTPs containing aa-UTP. After removal of unincorporated aa-dUTP and free amines, labeled cDNA was coupled to Cy3 or Cy5 mono-reactive dye (GE Healthcare). The *Francisella* microarray slides (Pathogen Functional Genomics Resource Center; PFGRC) contained 2331 70mer oligonucleotides in quadruplicates of the *F. tularensis* SchuS4 genome and several LVS genes as well as quadruplicates of 70mer oligonucleotides for 500 *Arabidopsis thaliana* as controls. Slides were prehybridized in 5x SSC, 10% SDS and 1% BSA, washed and then hybridized with cDNA probes at 42°C. After post-hybridization washes, the slides were scanned using the GenePix 4000B scanner and GenePix Pro v6.0 software. The microarray data were normalized using the TIGR MIDAS v2.22 and analyzed using the TIGR Multiexperiment Viewer v 4.2.1 (MeV) as part of the TM4 Suite software (45). In MeV, pooled, normalized Cy5/Cy3 intensities from wild-type LVS control arrays were compared to pooled, normalized Cy5/Cy3 intensities from LVSΔ*iclR* arrays. This list was filtered by statistical significance using

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Significance Analysis for Microarrays (SAM) provided on MeV after an 80% cut-off filter and using a false discovery rate of 5%.

Quantitative RT-PCR. Quantitative RT-PCR was performed in a 96-well format using the SensiMix™ SYBR & Fluorescein One-Step kit (Bioline) following the manufacturer's protocol. Briefly, 50 ng of RNA isolated from wild-type or *iclR* mutant strains was mixed with SensiMix™ SYBR & Fluorescein, RNase inhibitors, and designated primers in a 20 µl volume. A genomic DNA ladder and a no reverse transcriptase control were analyzed using the SensiMix™ SYBR & Fluorescein kit following the manufacturer's protocol with primers to *gyrA*. Thermocycling and detection was performed using the iCycler Thermal Cycler (Bio-Rad). All starting quantity (SQ) values were normalized to the mean SQ value for *gyrA*.

Antibiotic sensitivity assays. *F. tularensis* LVS was grown to mid-log phase in BHI broth supplemented with 1% IsoVitaleX, the bacterial suspension was spread onto chocolate agar plates, and antibiotic-containing filter paper discs were placed in the center of each plate. The rafampin (5µg), tetracycline (30µg), and colistin (10µg) were purchased pre-loaded from Becton Dickinson. The ampicillin and polymixin B discs were self-prepared by adding a 10 µl or 20 µl volume of antibiotic per disc at 10 µg ampicillin or 20 µg polymixin B. Bacteria were grown for 36 hr and the diameter of the zone of inhibition was measured.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Microarray data accession numbers. The raw and normalized microarray data is available on the GEO database under the following accession numbers: GSM574374, GSM574375, GSM574376, GSM574377, GSM574379, GSM574380, and GSE23454.

Results

Comparison of *iclR* alleles among *F. tularensis* subspecies and construction of *iclR* deletion mutants.

The locus FTL_1364 is annotated as a hypothetical protein in NCBI; however, some of its homologs in other *Francisella* species are annotated as proteins belonging to the IclR family of transcriptional regulators. A search for conserved domains found within FTL_1364 resulted in several related hits including a helix-turn-helix (HTH) domain conserved among IclR family members. Additionally, *Francisella* IclR has a C-terminal domain with high similarity to the IclR family profile Pfam01614. A recent publication describes a highly specific IclR family member profile that lies outside the HTH domain and covers less than 100 amino acids in the central region towards the C-terminal end (31). These authors classify current Pfam01614 members as belonging to the IclR family based on the new profile. Furthermore, BLAST analysis of *F. tularensis* LVS or SchuS4 IclR reveals high similarity to IclR family proteins found across many bacterial species. *F. tularensis* IclR proteins share considerable amino acid identity (30-40%) and amino acid similarity (60%) with non-*Francisella* IclR family proteins. Overall, the bioinformatic analysis strongly suggests that *Francisella* FTL_1364 and its

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

homologous loci in other *Francisella* species encode a protein belonging to the IclR family of transcriptional regulators.

Using NCBI and the *Francisella* genome browser (www.francisella.org) for annotations and synteny analysis, we found that the *iclR* locus has shared characteristics among *F. tularensis* subsp. *novicida* U112, *F. tularensis* subsp. *holarctica* LVS, and *F. tularensis* subsp. *tularensis* SchuS4 strains (FTN_0720, FTL_1364 and FTT_0748, respectively), (Figure 1A). On one side of *iclR* in each strain is a gene encoding a predicted protein with similarity to an esterase lipase (FTL_1363, FTN_0721, and FTT_0749). On the other side of *iclR* is a gene encoding a predicted protein with similarity to the multidrug efflux protein EmrA (FTL_1365-66, FTN_0718, and FTT_0747). One difference is that EmrA is divided into two ORFs in LVS. There are other differences in the length and coding sequences of this genetic region, including an additional open reading frame in U112 that encodes a predicted protein of unknown function FTN_0719. Nevertheless, in each strain, *iclR* is located in a similar region of the genome.

Additionally, *iclR* itself is highly conserved among the three *F. tularensis* strains U112, LVS and SchuS4. SchuS4 *iclR* has three nucleotide differences compared to *iclR* from LVS that translate into two amino acid differences, S22G and H78Y, between LVS and SchuS4 IclR. U112 *iclR* has 95 nucleotide differences compared to LVS *iclR* and 94 nucleotide changes compared to SchuS4 *iclR*. Although this results in a three nucleotide truncation of U112 *iclR*, there is 80% amino acid identity between U112 IclR and SchuS4 and LVS IclR proteins (Figure 1B). While these similarities suggest that IclR is conserved among the U112, LVS, and SchuS4, there are a sufficient number of differences to account for possible functional deviations between these

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

243 strains as well. Due to genetic similarity and the contribution of IclR to the virulence for *F.*
244 *tularensis* subsp. *novicida,* we investigated the potential contribution of IclR to the virulence of
245 *F. tularensis* subspecies *holarctica* and *tularensis*. To do this, we made a clean deletion of the
246 *iclR* gene in the *F. tularensis* subspecies *holarctica* LVS (LVSΔ*iclR*) and SchuS4 (SchuS4Δ*iclR*)
247 using SOE PCR and allelic exchange in LVS (FTL_1364). We also generated an *iclR*
248 complementation strain by expression of *iclR* on a low-copy shuttle vector.

249

250 **LVS and SchuS4 *iclR* deletion mutants are competent for intracellular replication.**

251

252 One method to assess the contribution of IclR to *F. tularensis* virulence is to determine what role
253 IclR plays in intracellular replication. We used gentamicin protection assays in the J774A.1
254 murine macrophage-like cell line and the TC-1 murine lung epithelial cell-like cell line to assess
255 intracellular replication by *iclR* deletion mutant strains. Both LVSΔ*iclR* and wild-type LVS
256 replicated approximately two logs by 24 hr in both J774A.1 and TC-1 cells (Figure 2A-B). We
257 also performed these assays in bone marrow-derived macrophages, and both wild-type LVS and
258 LVSΔ*iclR* replicated intracellularly in these cells (Figure 2C). Similarly, the intracellular
259 replication of SchuS4Δ*iclR* was similar to wild-type SchuS4 in J774A.1 cells (Figure 2D). These
260 results demonstrate that IclR is not required for intracellular replication of LVS or SchuS4 in
261 these cell types.

262

263 **LVSΔ*iclR* is not attenuated following intranasal or intradermal inoculation of mice.**

264

IAI Accepts published online ahead of print

Properties other than intracellular replication contribute to *F. tularensis* pathogenesis. We therefore determined whether IclR was required for LVS virulence *in vivo*. To test this, we used a mouse model of pulmonary tularemia in which we inoculated C57BL/6 mice i.n. with a lethal dose ($1x10^5$ CFU) of LVS or LVSΔ*iclR*. At 1, 3, 7 and 8 days post inoculation the lungs, liver and spleen were harvested to enumerate the bacterial organ burdens (Figure 3A). These initial experiments revealed that there were no differences in the organ burdens at 1 or 3 days post inoculation. At day 7, there appeared to be slight differences in the organ burdens in the liver and spleen, and by day 8 the organ burdens in the liver and spleen had not increased. These initial experiments suggested that LVSΔ*iclR* may demonstrate enhanced clearance in the mouse. This would correlate with previously published data demonstrating a decrease in competitive index in the spleen at 48 hr for the subsp. *novicida* U112 *iclR* deletion mutant compared to wild-type *novicida* U112 (53).

To further investigate the possibility of a more subtle phenotype of enhanced clearance, we used a low dose ($1x10^3$ CFU) i.n. inoculation of groups of six wild-type C57BL/6 mice with LVS or LVSΔ*iclR*. At days 1, 3, 7 and 10 post inoculation, we again harvested the lungs, liver and spleen to calculate the bacterial organ burdens. There were no significant differences between the bacterial organ burdens of LVSΔ*iclR* or wild-type LVS at any time point (Figure 3B). This suggests that LVSΔ*iclR* is not attenuated in a mouse model of pulmonary tularemia.

Since the experiments with subsp. *novicida* U112 *iclR* deletion mutant were performed using subcutaneous (s.c.) and i.p. inoculation, we investigated whether a role for *iclR* in pathogenesis may be route-specific. Groups of 6 to 7 wild-type C57BL/6 mice were infected i.d. with $3x10^5$

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

CFU of LVS or LVSΔ*iclR*. The i.d. route has a comparable LD_{50} dose and is similar in nature to the s.c. route (17). At 1, 3, and 7 days post inoculation, we again harvested the lungs, liver and spleen and determined bacterial organ burdens. At each time point and in each ogran, there was no significant difference in the bacterial burdens comparing LVS and LVSΔ*iclR* (Figure 3C). These data indicate that in LVS, *iclR* is not required for pathogenesis in the mouse via the i.n. or i.d. route.

SchuS4Δ*iclR* is not attenuated following intranasal inoculation of mice.

Although *iclR* does not appear to be required for LVS pathogenesis, it is possible that *iclR* plays a role in SchuS4 pathogenesis. We inoculated groups of four wild-type C57BL/6 mice i.n. with a lethal dose (100 CFU) of wild-type SchuS4 or SchuS4Δ*iclR*. At 1 and 3 days post inoculation, the lungs, liver and spleen were harvested to enumerate the bacterial organ burdens of infected mice (Figure 4). At both time points and in each organ, there were no differences in bacterial burden between wild-type SchuS4 and SchuS4Δ*iclR*. These data suggest that IclR does not play a role in the *in vivo* virulence of SchuS4 when assessed by the mouse model of pulmonary tularemia.

A subsp. *novicida* U112 *iclR* transposon mutant is attenuated following intranasal inoculation of mice.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

As noted above, an *iclR* deletion mutant in subsp. *novicida* U112 displays decreased competitive index in the spleen following s.c. and i.p inoculation of mice (53). Therefore, we wanted to determine whether *iclR* is required for subsp. *novicida* U112 pathogenesis in a pulmonary mouse model. We inoculated groups of six wild-type C57BL/6 mice i.n. with a dose of approximately 10 CFU of wild-type U112 or a U112 *iclR* transposon mutant. At 1 and 5 days post inoculation, the lungs, liver and spleen were harvested and the bacterial organ burdens were enumerated. Each organ had reduced burdens of the *iclR* transposon mutant compared to wild-type U112, and at day 5 these differences were statistically significant in the liver and spleen (Figure 5). These data suggest that *iclR* is required for U112 pathogenesis via the i.n. route and correlates with the previously published data using the s.c. and i.p. routes.

Deletion of *iclR* does not affect IL-1β expression or cytotoxicity of infected cells.

To determine if there is an altered cellular response to LVSΔ*iclR* compared to wild-type LVS, we measured the production of pro-inflammatory cytokines by infected cells. Bone marrow-derived macrophages were infected at an MOI 500 with LVS or LVSΔ*iclR* and the supernatants were analyzed for IL-1β at 24 hr post infection (Figure 6A). The levels IL-1β measured in the supernatants of LVSΔ*iclR*-infected cells was similar to that of cells infected with wild-type LVS, and no differences between strains were statistically significant.

F. tularensis is also reported to induce cytotoxicity of infected macrophages. To determine whether there was a change in cytotoxicity induced by LVSΔ*iclR*, we infected murine bone

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

marrow-derived macrophages with LVS or LVSΔ*iclR* at an MOI 500 and performed cytotoxicity assays on supernatants collected at 24 hr post infection. As shown in Figure 6B, LVSΔ*iclR* induces cytotoxicity in infected cells to a level similar to that of wild-type LVS, and no differences between strains were statistically significant.

The effects of IclR on gene expression.

Due to its homology to transcriptional regulators, we used microarray analysis to determine what genes in LVS were affected by IclR by comparing gene expression between the LVSΔ*iclR* mutant and wild-type LVS. We grew LVS and LVSΔ*iclR* to mid-log phase to harvest RNA for reverse transcription and amino-allyl labeling of cDNA, and the labeled cDNA was hybridized to microarray slides. The slides are printed for every annotated ORF for SchuS4, plus LVS alleles that are either not present or are variant in SchuS4, but they are not tailored to *F. tularensis* subsp. *novicida.* Three separate microarrays from independent RNA samples were pooled and statistically significant gene expression differences between LVSΔ*iclR* and wild-type LVS were determined by SAM (Table 1). Genes exhibiting significant changes in expression are listed by the provided locus annotations, LVS or SchuS4, as printed on the slides.

Using the above criteria, we identified 13 downregulated and 4 upregulated genes in LVSΔ*iclR*. The list of genes identified comprises diverse functional groups suggesting that IclR does not impact expression of one specific functional group of proteins. There were several IclR-affected genes annotated as encoding hypothetical proteins. To get a better idea of what types of proteins

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

these genes may be encoding and possibly obtain insight on IclR function, we performed BLASTp analyses. Many of the proteins were only conserved in *Francisella* with no similarity to proteins or conserved domains in other bacteria. However, there were several with similarity to known proteins in other bacteria and these are described in Table 1. Although most of the genes were represented exclusively by the SchuS4 allele, there were two cases where the SchuS4 and LVS homologs were both printed on the microarray slide and also appeared on the gene list as having significant expression changes in the absence of IclR. FTT_0741c and its FTL_1373 homolog were both upregulated in LVSΔ*iclR*, and both FTL_0388 and its homolog FTT_0885 were downregulated in LVSΔ*iclR*. Overall, although further studies need to be performed to demonstrate a function of IclR, both bioinformatic and microarray data suggest that *Francisella* IclR could function as a transcriptional regulator.

Using the above criteria, we identified 13 downregulated and 4 upregulated genes in LVSΔ*iclR*. The list of genes identified comprises diverse functional groups suggesting that IclR does not impact expression of one specific functional group of proteins. There were several affected genes annotated as encoding hypothetical proteins. To get a better idea of what types of proteins these genes may be encoding and possibly obtain insight on IclR function, we performed BLASTp analyses. Many of the proteins were only conserved in *Francisella* with no similarity to proteins or conserved domains in other bacteria. However, there were several with similarity to known proteins in other bacteria and these are described in Table 1. Although most of the genes did not have both the LVS and SchuS4 homologs printed on the slide, there were two cases where both the SchuS4 and LVS homologs appeared on the microarray list. FTT_0741c and its FTL_1373 homolog were both upregulated in LVSΔ*iclR*, and both FTL_0388 and its homolog FTT_0885

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

were downregulated in LVSΔ*iclR*. Overall, although further studies need to be performed to confirm the function of IclR, both bioinformatic and microarray data suggest that *Francisella* IclR could function as a transcriptional regulator.

Comparison of IclR-regulated genes between LVS, SchuS4 and U112.

One explanation for the phenotypic differences observed for *iclR* mutants among the *F. tularensis* U112, LVS and SchuS4 strains could be due to differences in the genes affected by IclR among the strains. To address this we performed a more detailed examination of the genes on our microarray list. First, we performed synteny analysis using the genome synteny tool at www.francisella.org to determine whether each gene was annotated in SchuS4, LVS and U112. We observed that there were a few genes that were not annotated or not present in all three strains, as shown in Table 1. Secondly, we generated alignments and protein translations of the genes using Vector NTI software based on the NCBI annotation or the putative loci of non-annotated genes from the synteny analysis, if they were found. For example, sequence alignments revealed that in LVS there is an unannotated ORF between FTL_1120 and FTL_1121 bearing homology to FTT_1082. Nearly half of the genes were similarly annotated and encoded one intact open reading frame (ORF) in SchuS4, LVS and U112. However, a significant percentage of genes displayed considerable sequence differences between strains as described in Table 1.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Of these genes, many were not intact in the virulent strains LVS and/or SchuS4, whereas the homologous genes in U112 were intact. For example, FTL_1506 and FTL_1507 are pseudogenes because they encode two ORFs while their SchuS4 (FTT_0723c) and U112 (FTN_0634) encode only one ORF. One special case is FTT0715, which along with its LVS homolog FTL_1521, has two large deletions, 131bp (119bp in LVS) and 197bp, when compared to the U112 homolog FTN_0627. The significance of these deletions cannot be inferred, and though these genes are not pseudogenes, the fact that these large deletions are present only in SchuS4 and LVS is noteworthy. This also highlights the fact that many of the intact genes on the microarray list have greater overall sequence differences between U112 and LVS or SchuS4 when compared to that of the differences between LVS and SchuS4.

We next wanted to determine whether the set of genes that were changed in expression in LVSΔ*iclR* were also changed in the absence of IclR in U112. First, we performed quantitative RT-PCR on six genes that were differentially-regulated in the microarray for LVS versus LVSΔ*iclR* (Figure 7A) and normalized to the housekeeping gene *gyrA*. We also included *iclR*. As expected, we detected a dramatic decrease in *iclR* transcript in LVSΔ*iclR* and negligible change in *gyrA*. Of the six genes analyzed, four repeated the trend seen in the microarray analysis. For the two genes that did not, the primers appeared to amplify with similar efficiencies to other primers (data not shown). Overall, the qRT-PCR data supports the fact that the genes identified in our microarray are changed in expression in the absence of IclR using a different method. We then tested the same set of gene homologs on RNA isolated from wild-type U112 and the U112 *iclR* transposon mutant (Figure 7B). Quantitative RT-PCR first verified that *iclR* transcripts were substantially lower in the transposon mutant. Overall, the six selected

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

genes appear to be changed similarly to their LVS homologs, suggesting a similar set of genes affected by IclR in U112. These analyses do not account for any additional genes affected by U112 IclR that were not affected by LVS IclR as detected by microarray. Furthermore, these analyses alone are not sufficient to extrapolate any correlations in terms of IclR function or which of the IclR-affected genes are likewise impacted at the protein level or functional.

The effects of IclR on antibiotic resistance.

Other IclR family proteins are known to be involved in the regulation of multi-drug efflux pumps (39). In all three *F. tularensis* subspecies, *iclR* is located near ORFs encoding hypothetical proteins that have homology to the EmrA multidrug efflux pump. In LVS, the two ORFs encoding proteins with EmrA homology that are found upstream of *iclR* were not changed in expression as determined by our microarray analysis. Nevertheless, the microarray data for LVSΔ*iclR* showed increased expression of a gene encoding a protein with homology to organic solvent tolerance proteins, suggesting that IclR may be involved in repression of some genes involved in drug efflux. Organic solvent tolerance is often associated with multi-drug efflux pumps, most notably in *Escherichia coli* and *Pseudomonas putida* (42). Furthermore, our BLASTp analyses of hypothetical genes that appear in the LVSΔ*iclR* microarray gene list also reveal proteins with homology to other transporter proteins. To determine whether *iclR* is involved for drug efflux, we performed disc diffusion assays using a panel of antibiotics. Antibiotics selected for analysis were chosen as representatives from several classes of antibiotics targeting cell wall synthesis, protein synthesis, nucleic acid synthesis, and cell

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

membrane integrity. There was no difference in antibiotic sensitivity between wild-type LVS and LVSΔ*iclR* using this method (Figure 8).

Discussion

Herein we investigated the contribution of the putative transcriptional regulator IclR to *F. tularensis* pathogenicity. In this study, we found that the LVSΔ*iclR* was not attenuated for intracellular replication in J774A.1 macrophage-like cells, TC-1 epithelial cells, or bone marrow-derived macrophages. Similarly, SchuS4Δ*iclR* was not attenuated for replication in J774A.1 cells. These data are consistent with published data by Weiss et al. for the *novicida* U112 *iclR* deletion mutant strain in bone-marrow derived macrophages (53).

When compared to wild-type LVS, LVSΔ*iclR* did not impact IL-1β induction or cytotoxicity of infected cells, which is different from that of the published *novicida* studies (53). It is important to note that the methods used for these analyses were different between the two studies. The levels of IL-1β that we reported in this study are near but not below the limit of detection for the ELISA. The fact that the levels of IL-1β induced are low is consistent with other studies evidencing that LVS suppresses the inflammatory response (26, 50). Furthermore, Weiss et al. used pre-stimulated bone marrow-derived macrophages, whereas we used naïve bone marrow-derived macrophages. Macrophages pre-treated with LPS or heat-killed *F. tularensis* subp. *novicida* as well as thioglycolate-elicited macrophages produce higher levels of IL-1β in

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

response to infection (11, 12, 36, 52). Another possibility is that there are strain-specific differences in the role of IclR, as evidenced by the results of the *in vivo* studies discussed below.

Unlike the *novicida iclR* deletion mutant, neither LVSΔ*iclR* nor SchuS4Δ*iclR* were attenuated in mice following i.n. inoculation. There were differences in the experimental design between our studies and the *novicida* study. We initially inoculated mice i.n. and monitored lung, liver, and spleen over several days post infection. Weiss et al. used s.c. and i.p. inoculations in competition assays examining the spleen at 2 days post infection. It is possible that inoculation route may have an impact on the importance of IclR on establishing infection. To address the possibility that the phenotype is route-specific, we performed a reciprocal analysis by evaluating the virulence of LVS and U112 *iclR* mutants in i.d. and i.n. infection models, respectively. The results confirmed that the *Francisella* virulence-specific properties of IclR are restricted to subspecies *novicida.*

It is not clear why IclR is required for virulence in U112 but not LVS and SchuS4. Based on our microarray analysis, the subspecies-specific sequence differences among IclR-affected genes could contribute to the functional differences we observe for IclR between subspecies. Many of the genes are intact in U112, but in LVS and/or SchuS4, the homologous genes are pseudogenes or displayed significant sequence variation (e.g. two large deletions in FTT0715/FTL_1521). The virulent subspecies of *F. tularensis* are noted for their genome decay as characterized by smaller genomes as well as increased numbers of pseudogenes, transposases and gene rearrangements (51). Genome-wide analyses of *Francisella* strains support this idea and many of the genes changed in LVSΔ*iclR* that we identified to be pseudogenes correlate with those found in other

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

studies (7, 44). It is possible that IclR in subspecies *novicida* exerts its effects on genes that are intact whereas in LVS and SchuS4, IclR affects genes that are similar those in *novicida* but because of disruptions or changes to the ORFs, many of these genes are transcribed but do not encode functional proteins. We must also consider that there are two genes in the list that are absent in U112 that are present in LVS and SchuS4, and the absence of a gene affected by IclR in *novicida* could also contribute to the different phenotypes. Overall, analysis of the genes identified in our microarray suggest that the majority of genes affected by IclR have differences in sequence between the three subspecies and that this variation could contribute to the phenotypic disparities observed.

Taken together, our data suggest that IclR contributes to the virulence of U112 but not to that of LVS or SchuS4, highlighting the fact that there are significant differences among these strains. Another example of differences among strains is seen in the conserved acid phosphatases AcpA, AcpB, and AcpC. These proteins were shown to be required for the virulence of subsp. *novicida*, but not for the virulence of SchuS4 (8, 38). Even though IclR may not play a major role in SchuS4 or LVS virulence, there are other potential roles that IclR could be have as a functional transcriptional regulator. Quite a few of the microarray-identified genes encode hypothetical proteins, but there are others that encode proteins with known functions or are homologous to proteins with known functions. Investigation into these proteins may provide an additional understanding of the function of IclR in *F. tularensis.* For example, in *Pseudomonas putida*, the IclR family proteins TtgT and TtgV regulate operons encoding genes that form efflux pumps for organic solvent extrusion (16, 22). Although our antibiotic sensitivity assays showed no role for IclR in drug efflux by LVS, we cannot rule out the involvement of IclR in the regulation of a

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

system specific for organic solvent efflux or the role of IclR in drug efflux in other *F. tularensis* subspecies. Finally, direct comparison of the complete transcriptional profiles of subspecies *novicida*, *tularensis* and *holarctica iclR* deletion strains might reveal some clues to the properties that are responsible for the phenotypic differences. Unfortunately, the currently available microarrays do not contain targets for genes found exclusively in subspecies *novicida*.

Acknowledgments

This work was supported by NIH grants #U54 AI057157 (JT and TK) from the Southeastern Regional Center of Excellence for Emerging Infections and Biodefense and R56 AI069339 (TK). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. The *Francisella* microarrays were obtained through NIAID's Pathogen Functional Genomics Resource Center, managed and funded by Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS and operated by the J. Craig Venter Institute. A special thanks to Eric LoVullo, Jason Brunton, and Shaun Steele for their assistance performing the animal experiments and to John C. Braisted and Jianwei Li (J. Craig Venter Institute) for their assistance in using the TM4 Suite software.

References

1. Anthony, L. D., R. D. Burke, and F. E. Nano. 1991. Growth of Francisella spp. in rodent macrophages. Infect. Immun. **59:**3291-3296.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

530 2. **Birdsell, D. N., T. Stewart, A. J. Vogler, E. Lawaczeck, A. Diggs, T. L. Sylvester, J. L.**
531 **Buchhagen, R. K. Auerbach, P. Keim, and D. M. Wagner.** 2009. Francisella tularensis subsp.
532 novicida isolated from a human in Arizona. BMC Res. Notes. **2:**223. doi: 10.1186/1756-0500-2-
533 223.

534 3. **Bosio, C. M., and S. W. Dow.** 2005. Francisella tularensis induces aberrant activation of
535 pulmonary dendritic cells. J. Immunol. **175:**6792-6801.

536 4. **Centers for Disease Control and Prevention (CDC).** 2005. Tularemia transmitted by insect
537 bites--Wyoming, 2001-2003. MMWR Morb. Mortal. Wkly. Rep. **54:**170-173.

538 5. **Centers for Disease Control and Prevention (CDC).** 2009. Tularemia - Missouri, 2000-
539 2007. MMWR Morb. Mortal. Wkly. Rep. **58:**744-748.

540 6. **CHAMBERLAIN, R. E.** 1965. Evaluation of Live Tularemia Vaccine Prepared in a
541 Chemically Defined Medium. Appl. Microbiol. **13:**232-235.

542 7. **Champion, M. D., Q. Zeng, E. B. Nix, F. E. Nano, P. Keim, C. D. Kodira, M. Borowsky,**
543 **S. Young, M. Koehrsen, R. Engels, M. Pearson, C. Howarth, L. Larson, J. White, L.**
544 **Alvarado, M. Forsman, S. W. Bearden, A. Sjostedt, R. Titball, S. L. Michell, B. Birren, and**
545 **J. Galagan.** 2009. Comparative genomic characterization of Francisella tularensis strains
546 belonging to low and high virulence subspecies. PLoS Pathog. **5:**e1000459. doi:
547 10.1371/journal.ppat.1000459.

548 8. **Child, R., T. D. Wehrly, D. Rockx-Brouwer, D. W. Dorward, and J. Celli.** 2009. Acid
549 phosphatases do not contribute to pathogenesis of Type A Francisella tularensis. Infect. Immun. .
550 doi: 10.1128/IAI.00965-09.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

551 9. **Clarridge, J. E.,3rd, T. J. Raich, A. Sjosted, G. Sandstrom, R. O. Darouiche, R. M.**
552 **Shawar, P. R. Georghiou, C. Osting, and L. Vo.** 1996. Characterization of two unusual
553 clinically significant Francisella strains. J. Clin. Microbiol. **34:**1995-2000.

554 10. **Clemens, D. L., B. Y. Lee, and M. A. Horwitz.** 2004. Virulent and avirulent strains of
555 Francisella tularensis prevent acidification and maturation of their phagosomes and escape into
556 the cytoplasm in human macrophages. Infect. Immun. **72:**3204-3217. doi:
557 10.1128/IAI.72.6.3204-3217.2004.

558 11. **Cole, L. E., K. L. Elkins, S. M. Michalek, N. Qureshi, L. J. Eaton, P. Rallabhandi, N.**
559 **Cuesta, and S. N. Vogel.** 2006. Immunologic consequences of Francisella tularensis live
560 vaccine strain infection: role of the innate immune response in infection and immunity. J.
561 Immunol. **176:**6888-6899.

562 12. **Cole, L. E., A. Santiago, E. Barry, T. J. Kang, K. A. Shirey, Z. J. Roberts, K. L. Elkins,**
563 **A. S. Cross, and S. N. Vogel.** 2008. Macrophage proinflammatory response to Francisella
564 tularensis live vaccine strain requires coordination of multiple signaling pathways. J. Immunol.
565 **180:**6885-6891.

566 13. **Cowley, S. C., C. J. Gray, and F. E. Nano.** 2000. Isolation and characterization of
567 Francisella novicida mutants defective in lipopolysaccharide biosynthesis. FEMS Microbiol.
568 Lett. **182:**63-67.

569 14. **Deng, K., R. J. Blick, W. Liu, and E. J. Hansen.** 2006. Identification of Francisella
570 tularensis genes affected by iron limitation. Infect. Immun. **74:**4224-4236. doi:
571 10.1128/IAI.01975-05.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

15. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, K. Tonat, and Working Group on Civilian Biodefense.** 2001. Tularemia as a biological weapon: medical and public health management. JAMA. **285:**2763-2773.

16. **Duque, E., A. Segura, G. Mosqueda, and J. L. Ramos.** 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of Pseudomonas putida. Mol. Microbiol. **39:**1100-1106.

17. **Elkins, K. L., R. K. Winegar, C. A. Nacy, and A. H. Fortier.** 1992. Introduction of Francisella tularensis at skin sites induces resistance to infection and generation of protective immunity. Microb. Pathog. **13:**417-421.

18. **Feldman, K. A., R. E. Enscore, S. L. Lathrop, B. T. Matyas, M. McGuill, M. E. Schriefer, D. Stiles-Enos, D. T. Dennis, L. R. Petersen, and E. B. Hayes.** 2001. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N. Engl. J. Med. **345:**1601-1606.

19. **Fortier, A. H., M. V. Slayter, R. Ziemba, M. S. Meltzer, and C. A. Nacy.** 1991. Live vaccine strain of Francisella tularensis: infection and immunity in mice. Infect. Immun. **59:**2922-2928.

20. **Fuller, J. R., R. R. Craven, J. D. Hall, T. M. Kijek, S. Taft-Benz, and T. H. Kawula.** 2008. RipA, a cytoplasmic membrane protein conserved among Francisella species, is required for intracellular survival. Infect. Immun. **76:**4934-4943. doi: 10.1128/IAI.00475-08.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

592 21. **Gallagher, L. A., E. Ramage, M. A. Jacobs, R. Kaul, M. Brittnacher, and C. Manoil.**
593 2007. A comprehensive transposon mutant library of Francisella novicida, a bioweapon
594 surrogate. Proc. Natl. Acad. Sci. U. S. A. **104:**1009-1014. doi: 10.1073/pnas.0606713104.

595 22. **Guazzaroni, M. E., W. Teran, X. Zhang, M. T. Gallegos, and J. L. Ramos.** 2004. TtgV
596 bound to a complex operator site represses transcription of the promoter for the multidrug and
597 solvent extrusion TtgGHI pump. J. Bacteriol. **186:**2921-2927.

598 23. **Hall, J. D., R. R. Craven, J. R. Fuller, R. J. Pickles, and T. H. Kawula.** 2007. Francisella
599 tularensis replicates within alveolar type II epithelial cells in vitro and in vivo following
600 inhalation. Infect. Immun. **75:**1034-1039. doi: 10.1128/IAI.01254-06.

601 24. **Hollis, D. G., R. E. Weaver, A. G. Steigerwalt, J. D. Wenger, C. W. Moss, and D. J.**
602 **Brenner.** 1989. Francisella philomiragia comb. nov. (formerly Yersinia philomiragia) and
603 Francisella tularensis biogroup novicida (formerly Francisella novicida) associated with human
604 disease. J. Clin. Microbiol. **27:**1601-1608.

605 25. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering
606 hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene.
607 **77:**61-68.

608 26. **Huang, M. T., B. M. Mortensen, D. J. Taxman, R. R. Craven, S. Taft-Benz, T. M. Kijek,**
609 **J. R. Fuller, B. K. Davis, I. C. Allen, W. Brickey, D. Gris, H. Wen, T. H. Kawula, and J. P.**
610 **Ting.** Deletion of *ripA* alleviates suppression of the inflammasome and MAP kinase by
611 *Francisella tularensis*. J. Immunol., in press.

612 27. **Kadzhaev, K., C. Zingmark, I. Golovliov, M. Bolanowski, H. Shen, W. Conlan, and A.**
613 **Sjostedt.** 2009. Identification of genes contributing to the virulence of Francisella tularensis

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

SCHU S4 in a mouse intradermal infection model. PLoS One. **4:**e5463. doi: 10.1371/journal.pone.0005463.

28. **Kandemir, B., I. Erayman, M. Bitirgen, E. T. Aribas, and S. Guler.** 2007. Tularemia presenting with tonsillopharyngitis and cervical lymphadenitis: report of two cases. Scand. J. Infect. Dis. **39:**620-622. doi: 10.1080/00365540601105814.

29. **Keim, P., A. Johansson, and D. M. Wagner.** 2007. Molecular epidemiology, evolution, and ecology of Francisella. Ann. N. Y. Acad. Sci. **1105:**30-66. doi: 10.1196/annals.1409.011.

30. **Kraemer, P. S., A. Mitchell, M. R. Pelletier, L. A. Gallagher, M. Wasnick, L. Rohmer, M. J. Brittnacher, C. Manoil, S. J. Skerett, and N. R. Salama.** 2009. Genome-wide screen in Francisella novicida for genes required for pulmonary and systemic infection in mice. Infect. Immun. **77:**232-244. doi: 10.1128/IAI.00978-08.

31. **Krell, T., A. J. Molina-Henares, and J. L. Ramos.** 2006. The IclR family of transcriptional activators and repressors can be defined by a single profile. Protein Sci. **15:**1207-1213. doi: 10.1110/ps.051857206.

32. **Leelaporn, A., S. Yongyod, S. Limsrivanichakorn, T. Yungyuen, and P. Kiratisin.** 2008. Francisella novicida bacteremia, Thailand. Emerg. Infect. Dis. **14:**1935-1937.

33. **LoVullo, E. D., L. A. Sherrill, L. L. Perez, and M. S. Pavelka Jr.** 2006. Genetic tools for highly pathogenic Francisella tularensis subsp. tularensis. Microbiology. **152:**3425-3435. doi: 10.1099/mic.0.29121-0.

34. **Lubbert, C., C. Taege, T. Seufferlein, and R. Grunow.** 2009. Prolonged course of tick-borne ulceroglandular tularemia in a 20-year-old patient in Germany--case report and review of the literature. Dtsch. Med. Wochenschr. **134:**1405-1410. doi: 10.1055/s-0029-1225296.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

636 35. **Maier, T. M., M. S. Casey, R. H. Becker, C. W. Dorsey, E. M. Glass, N. Maltsev, T. C.**
637 **Zahrt, and D. W. Frank.** 2007. Identification of Francisella tularensis Himar1-based transposon
638 mutants defective for replication in macrophages. Infect. Immun. **75:**5376-5389. doi:
639 10.1128/IAI.00238-07.

640 36. **Mariathasan, S., D. S. Weiss, V. M. Dixit, and D. M. Monack.** 2005. Innate immunity
641 against Francisella tularensis is dependent on the ASC/caspase-1 axis. J. Exp. Med. **202:**1043-
642 1049. doi: 10.1084/jem.20050977.

643 37. **McCaffrey, R. L., and L. A. Allen.** 2006. Francisella tularensis LVS evades killing by
644 human neutrophils via inhibition of the respiratory burst and phagosome escape. J. Leukoc. Biol.
645 **80:**1224-1230. doi: 10.1189/jlb.0406287.

646 38. **Mohapatra, N. P., S. Soni, T. J. Reilly, J. Liu, K. E. Klose, and J. S. Gunn.** 2008.
647 Combined deletion of four Francisella novicida acid phosphatases attenuates virulence and
648 macrophage vacuolar escape. Infect. Immun. **76:**3690-3699. doi: 10.1128/IAI.00262-08.

649 39. **Molina-Henares, A. J., T. Krell, M. Eugenia Guazzaroni, A. Segura, and J. L. Ramos.**
650 2006. Members of the IclR family of bacterial transcriptional regulators function as activators
651 and/or repressors. FEMS Microbiol. Rev. **30:**157-186. doi: 10.1111/j.1574-6976.2005.00008.x.

652 40. **Ojeda, S. S., Z. J. Wang, C. A. Mares, T. A. Chang, Q. Li, E. G. Morris, P. A. Jerabek,**
653 **and J. M. Teale.** 2008. Rapid dissemination of Francisella tularensis and the effect of route of
654 infection. BMC Microbiol. **8:**215. doi: 10.1186/1471-2180-8-215.

655 41. **Qin, A., and B. J. Mann.** 2006. Identification of transposon insertion mutants of Francisella
656 tularensis tularensis strain Schu S4 deficient in intracellular replication in the hepatic cell line
657 HepG2. BMC Microbiol. **6:**69. doi: 10.1186/1471-2180-6-69.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

658 42. **Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-Gonzalez, A. Rojas, W.**
659 **Teran, and A. Segura.** 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Annu.
660 Rev. Microbiol. **56:**743-768. doi: 10.1146/annurev.micro.56.012302.161038.

661 43. **Reintjes, R., I. Dedushaj, A. Gjini, T. R. Jorgensen, B. Cotter, A. Lieftucht, F.**
662 **D'Ancona, D. T. Dennis, M. A. Kosoy, G. Mulliqi-Osmani, R. Grunow, A. Kalaveshi, L.**
663 **Gashi, and I. Humolli.** 2002. Tularemia outbreak investigation in Kosovo: case control and
664 environmental studies. Emerg. Infect. Dis. **8:**69-73.

665 44. **Rohmer, L., C. Fong, S. Abmayr, M. Wasnick, T. J. Larson Freeman, M. Radey, T.**
666 **Guina, K. Svensson, H. S. Hayden, M. Jacobs, L. A. Gallagher, C. Manoil, R. K. Ernst, B.**
667 **Drees, D. Buckley, E. Haugen, D. Bovee, Y. Zhou, J. Chang, R. Levy, R. Lim, W. Gillett, D.**
668 **Guenthener, A. Kang, S. A. Shaffer, G. Taylor, J. Chen, B. Gallis, D. A. D'Argenio, M.**
669 **Forsman, M. V. Olson, D. R. Goodlett, R. Kaul, S. I. Miller, and M. J. Brittnacher.** 2007.
670 Comparison of Francisella tularensis genomes reveals evolutionary events associated with the
671 emergence of human pathogenic strains. Genome Biol. **8:**R102. doi: 10.1186/gb-2007-8-6-r102.

672 45. **Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa,**
673 **T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E.**
674 **Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush.** 2003.
675 TM4: a free, open-source system for microarray data management and analysis. BioTechniques.
676 **34:**374-378.

677 46. **SASLAW, S., H. T. EIGELSBACH, J. A. PRIOR, H. E. WILSON, and S. CARHART.**
678 1961. Tularemia vaccine study. II. Respiratory challenge. Arch. Intern. Med. **107:**702-714.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

679 47. **Schulert, G. S., R. L. McCaffrey, B. W. Buchan, S. R. Lindemann, C. Hollenback, B. D.**
680 **Jones, and L. A. Allen.** 2009. Francisella tularensis genes required for inhibition of the
681 neutrophil respiratory burst and intramacrophage growth identified by random transposon
682 mutagenesis of strain LVS. Infect. Immun. **77:**1324-1336. doi: 10.1128/IAI.01318-08.

683 48. **Siret, V., D. Barataud, M. Prat, V. Vaillant, S. Ansart, A. Le Coustumier, J. Vaissaire,**
684 **F. Raffi, M. Garre, and I. Capek.** 2006. An outbreak of airborne tularaemia in France, August
685 2004. Euro Surveill. **11:**58-60.

686 49. **Su, J., J. Yang, D. Zhao, T. H. Kawula, J. A. Banas, and J. R. Zhang.** 2007. Genome-
687 wide identification of Francisella tularensis virulence determinants. Infect. Immun. **75:**3089-
688 3101. doi: 10.1128/IAI.01865-06.

689 50. **Telepnev, M., I. Golovliov, T. Grundstrom, A. Tarnvik, and A. Sjostedt.** 2003.
690 Francisella tularensis inhibits Toll-like receptor-mediated activation of intracellular signalling
691 and secretion of TNF-alpha and IL-1 from murine macrophages. Cell. Microbiol. **5:**41-51.

692 51. **Titball, R. W., and J. F. Petrosino.** 2007. Francisella tularensis genomics and proteomics.
693 Ann. N. Y. Acad. Sci. **1105:**98-121. doi: 10.1196/annals.1409.015.

694 52. **Ulland, T. K., B. W. Buchan, M. R. Ketterer, T. Fernandes-Alnemri, D. K. Meyerholz,**
695 **M. A. Apicella, E. S. Alnemri, B. D. Jones, W. M. Nauseef, and F. S. Sutterwala.** 2010.
696 Cutting Edge: Mutation of Francisella tularensis mviN Leads to Increased Macrophage Absent in
697 Melanoma 2 Inflammasome Activation and a Loss of Virulence. J. Immunol. . doi:
698 10.4049/jimmunol.1001610.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

699 53. **Weiss, D. S., A. Brotcke, T. Henry, J. J. Margolis, K. Chan, and D. M. Monack.** 2007. In
700 vivo negative selection screen identifies genes required for Francisella virulence. Proc. Natl.
701 Acad. Sci. U. S. A. **104:**6037-6042. doi: 10.1073/pnas.0609675104.

702

703 **Figure Legends**

704

705 **Figure 1. Comparison of *iclR* in three *Francisella* strains.** (A) Synteny diagram of the
706 genomic organization of the *iclR* locus in *F. tularensis* subspecies *novicida* U112 (FTN_0720),
707 *F. tularensis* subspecies *holarctica* LVS (FTL_1364), and *F. tularensis* subspecies *tularensis*
708 SchuS4 (FTT_0748). (B) Amino acid sequence alignment of *F. tularensis* subspeices *novicida*
709 U112, *F. tularensis* subspecies *holarctica* LVS, and *F. tularensis* subspecies *tularensis* SchuS4
710 IclR. Alignment was created using VectorNTI software and *iclR* sequences uploaded from NCBI
711 annotated genomes of each strain and translated using VectorNTI. Red letters highlight residues
712 conserved between all three strains. Blue letters highlight the residues conserved between two
713 strains.

714

715 **Figure 2. Intracellular replication of LVSΔ*iclR* and SchuS4Δ*iclR* in murine macrophages**
716 **or lung epithelial cells.** Gentamicin protection assays were performed by infecting (A) J774A.1
717 murine macrophages, (B) TC-1 murine lung epithelial cells, and (C) bone marrow-derived
718 macrophages with wild-type LVS or LVSΔ*iclR* at an MOI 100. (D) Gentamicin protection assay
719 was performed using J774A.1 cells infected with wild-type SchuS4 or SchuS4Δ*iclR*. Bars

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

represent the standard deviation of three replicate wells and each graph is representative of two separate experiments.

Figure 3. Recovery of LVSΔ*iclR* mutant in mice following i.n. or i.d. inoculation. C57BL/6 mice were inoculated with either wild-type LVS (circles) or LVSΔ*iclR* (triangles) i.n. at (A) a lethal dose of ~$1x10^5$ CFU or (B) a low dose of ~$1x10^3$ CFU. (C) C57BL/6 mice were inoculated with either wild-type LVS (circles) or LVSΔ*iclR* (triangles) i.d. at a dose of ~$3x10^5$ CFU. Each symbol represents data from a single mouse. There were no significant differences in recovery of mutant versus wild-type organisms from any organ at any time point as determined by the Mann-Whitney nonparametric test in the low dose (B) and i.d. (C) experiments.

Figure 4. Recovery of SchuS4Δ*iclR* mutant in mice following i.n. inoculation. C57BL/6 mice were inoculated with either wild-type SchuS4 (circles) or SchuS4Δ*iclR* (triangles) i.n. at a dose of ~100 CFU. No differences in recovery of mutant versus wild-type organisms from any organ at any time point were significant using the Mann-Whitney nonparametric test.

Figure 5. Recovery of U112 *iclR* transposon mutant in mice following i.n. inoculation. C57BL/6 mice were inoculated with either wild-type U112 (circles) or U112 *iclR* mutant (triangles) i.n. at a dose of ~10 CFU. Differences in recovery of mutant versus wild-type organisms at day 5 for the liver and spleen were significant using the Mann-Whitney nonparametric test.

IAI Accepts published online ahead of print

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Figure 6. IL-1β release and cytotoxicity in murine bone marrow-derived macrophages infection with LVSΔ*iclR*. Infections were carried out at an MOI 500 for wild-type LVS, LVSΔ*iclR*, and LVSΔ*iclR* + IclR (complementation). (A) IL-1β was quantified via ELISA and (B) cytotoxicity was quantified via ToxiLight bioassay (Lonza), both at 24hr post infection. Graphs are representative of at least three separate experiments, with duplicate or triplicate wells for each strain per experiment. No differences were significant by any strain comparison using the student's t-test.

Figure 7. Transcript levels of genes found significantly changed in microarray analysis comparing LVS and LVSΔ*iclR*. RNA was isolated from (A) wild-type *F. tularensis* subsp. *holarctica* LVS and LVSΔ*iclR* or (B) wild-type *F. tularensis* subspecies *novicida* U112 and a U112 *iclR* transposon mutant and used in qRT-PCR analysis for several genes that were significantly changed in the microarray. Data is presented as relative expression of log change in wild-type over the respective *iclR* mutant after normalization to *gyrA*. Graph is representative of two or three experiments.

Figure 8. Antibiotic sensitivity of LVSΔ*iclR*. Wild-type LVS and LVSΔ*iclR* were grown to mid-log phase, bacteria spread on chocolate agar, and an antibiotic-containing paper disc was added to the center. Bacteria were grown for 36 hr and the diameter of the zone of inhibition was measured. Experiment was performed in triplicate and the averages and standard deviations were calculated.

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

Table 1. Microarray gene expression in LVSΔ*iclR*

Gene Locus	Fold Change	Description- Annotation/BLASTp*	Gene comparison between strains □		
Downregulated			SchuS4	LVS	U112
FTT0748	34.57	*iclR*	Intact	Intact	Intact
FTT0980	5.02	Hypothetical protein; Aminotransferase class II	Intact	Intact	Intact
FTT0987	2.84	Hypothetical protein; membrane protein of unknown function	Pseudogene	Pseudogene	Intact
FTL_1506	2.82	Short-chain dehydrogenase/reductase family protein	Intact	Pseudogene	Intact
FTT1082	2.64	T1082 protein	Intact	Intact	Intact
FTL_0388/ FTT0885	2.63/2.18	Cation transporter; cobalt zinc cadmium cation transporter	Pseudogene	Intact	Intact
FTL_1256	2.62	Pseudogene; carbon-nitrogen hydrolase family protein	Pseudogene	Pseudogene	Intact
FTL_1507	2.57	3-oxoacyl-[acyl-carrier protein] reductase	Intact	Pseudogene	Intact
FTT1081c	2.53	Hypothetical protein; hemolysin-type binding protein	Intact	Intact	Absent
FTT1507	2.37	Hypothetical protein; thymosin beta-4 family protein	Intact	Absent	Intact
FTL_1122	2.19	Hypothetical membrane protein	Intact	Pseudogene	Absent
FTT0715	2.19	Chitinase family 18 protein	Two large deletions	Two large deletions	Intact
FTT0389	2.16	Acetyltransferase	Intact†	Intact†	Intact†
FTT0203c	1.97	Bifunctional purine biosynthesis protein (*purH*)	Intact	Intact	Intact
Upregulated					
FTL_1373/ FTT0741c	4.28/6.40	Organic solvent tolerance protein	Pseudogene	Pseudogene	Intact
FTT1555c	1.93	Ribonuclease III (*rnc*)	Intact	Intact	Intact
FTT1554c	1.88	tRNA pseudouridine synthetase B (*truB*)	Intact	Intact	Intact
FTT0554	1.59	Hypothetical protein	Intact	Intact	Intact

*Annotation from microarray; included BLASTp results when provided additional information

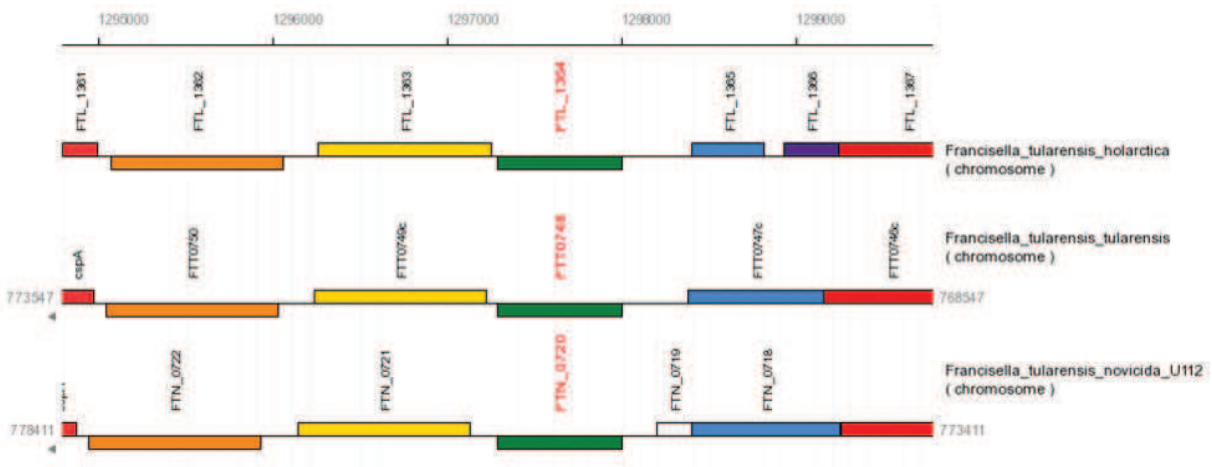
□ Following alignments, genes were designated as intact, absent, or a pseudogene (introduced stop codon/two shorter predicted ORFs); those without these designations are discussed in the text.

†Possible alternative start sites (details in text)

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

A



B

```
                1                                                  50
FTN_0720     (1) MSEDKRIQVISRAVNVLKSIGSKPDGMSLGEIAKQIYLPRSTVQRIVAAL
FTL_1364     (1) MSEDKRIQVISRAVNVLKSIGSKPDGMSLGEIAKQIYLPRSTVQRIVAAL
FTT_0748     (1) MSEDKRIQVISRAVNVLKSIGGKPDGMSLGEIAKQIYLPRSTVQRIVAAL
                51                                                 100
FTN_0720    (51) EEEGFIRSEGAGKILLGPGIFKLISSCYVDIITLTQDSLSKLSEKVQETV
FTL_1364    (51) EEEGFIKSEGAGKILLGPGIFKLISSSHADIISLTQDSLSKLSAKIQETV
FTT_0748    (51) EEEGFIKSEGAGKILLGPGIFKLISSSYADIISLTQDSLSKLSAKIQETV
               101                                                 150
FTN_0720   (101) SLTQSYDKNLLVLHRFVVNRELQIIPRVGALVPLHKTAAGRALLSLHTDD
FTL_1364   (101) VITQPYDSDLLIVHRFIINRELQVLPRVGALIPIYNSSSGRAILALHTDD
FTT_0748   (101) VITQPYDSDLLIVHRFIINRELQVLPRVGALIPIYNSSSGRAILALHTDD
               151                                                 200
FTN_0720   (151) KIIKILGEQITDDKELFEKIAKIRVKGIDIDYSQTIKGIVTIGVAIKSFL
FTL_1364   (151) KIIEILGEQIPDDKELFEKIAKIRADGIEFDYGKVIKGIVSIGVAINTFL
FTT_0748   (151) KIIEILGEQIPDDKELFEKIAKIRADGIEFDYGKVIKGIVSIGVAINTFL
               201                                    240
FTN_0720   (201) GDFGVSILAPQHRFDQNKDLYVDELLKVKSKIIDEIGV--
FTL_1364   (201) GTFGISILTPQHRFDKYKEFYVNELLKTKSKIIDEIGIK-
FTT_0748   (201) GTFGISILTPQHRFDKYKEFYVNELLKTKSKIIDEIGIK-
```

Figure 1

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

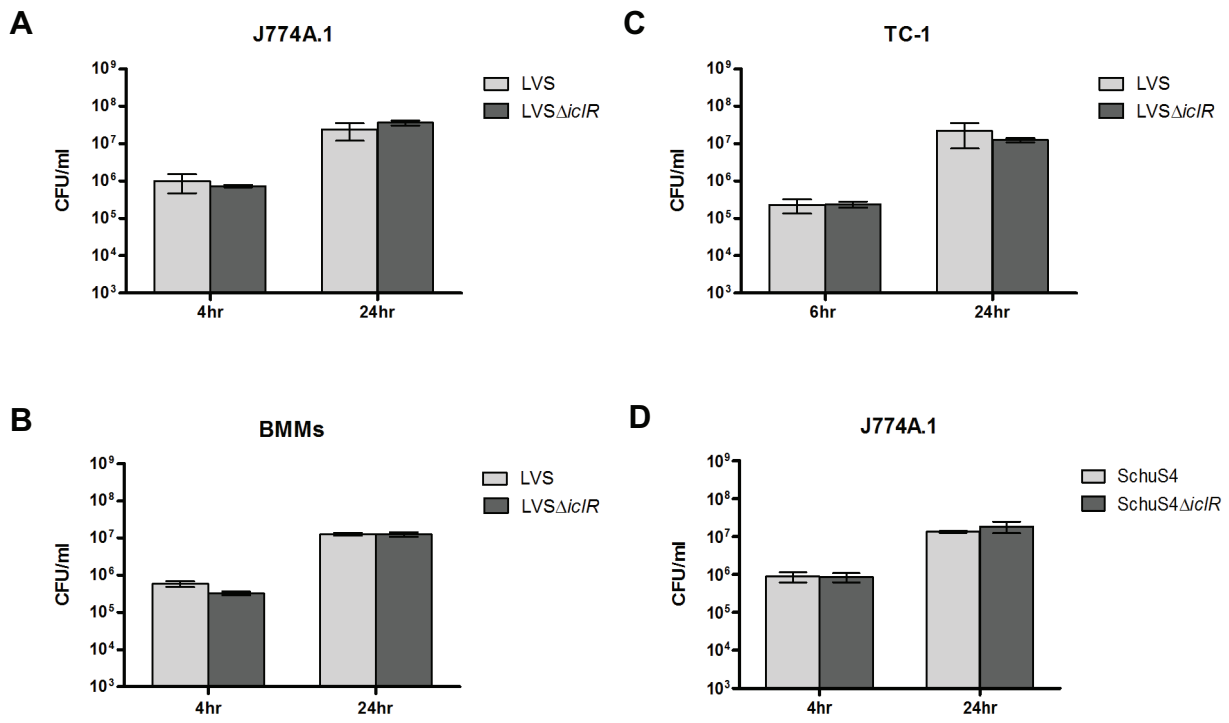


Figure 2

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

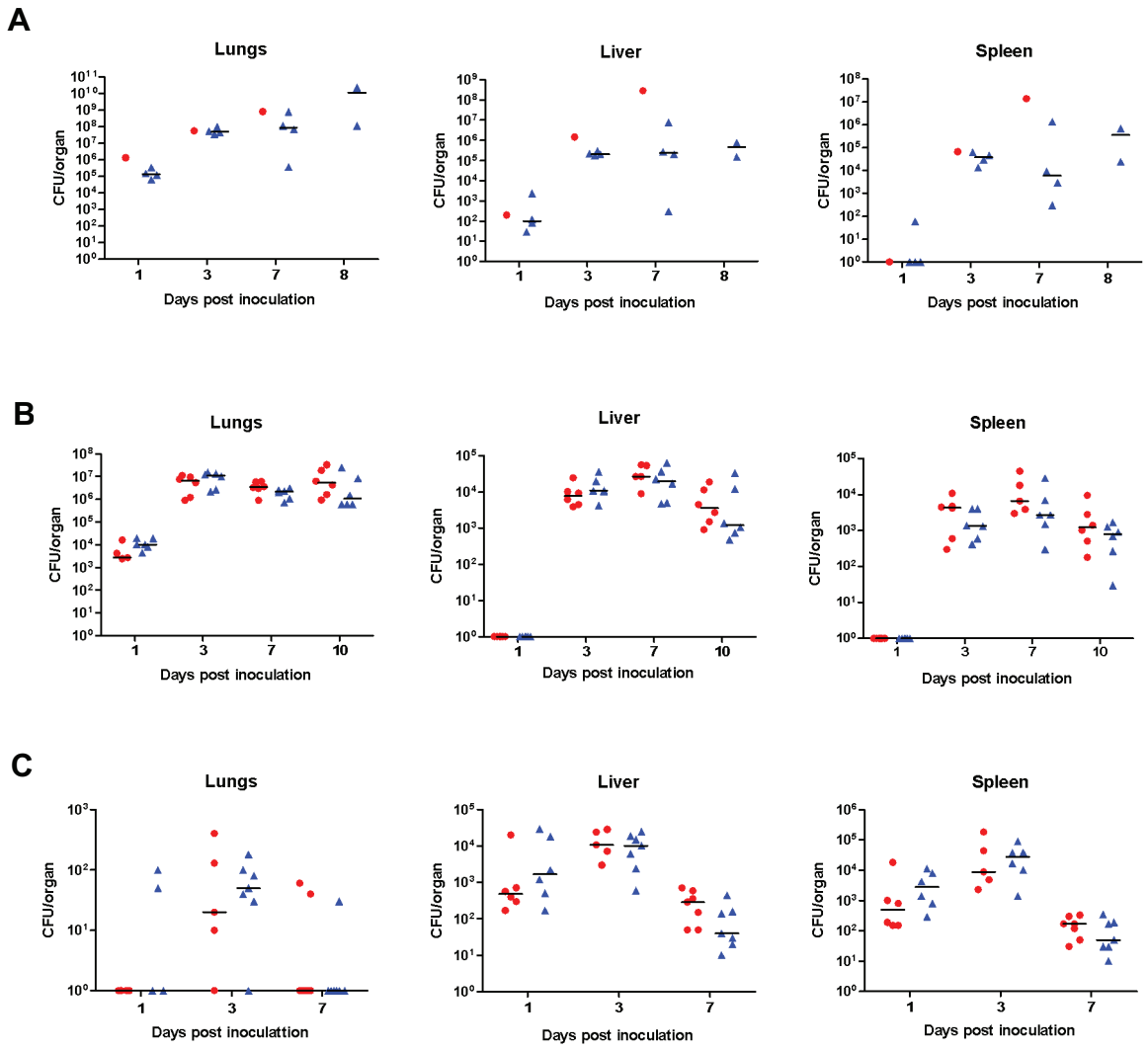


Figure 3

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

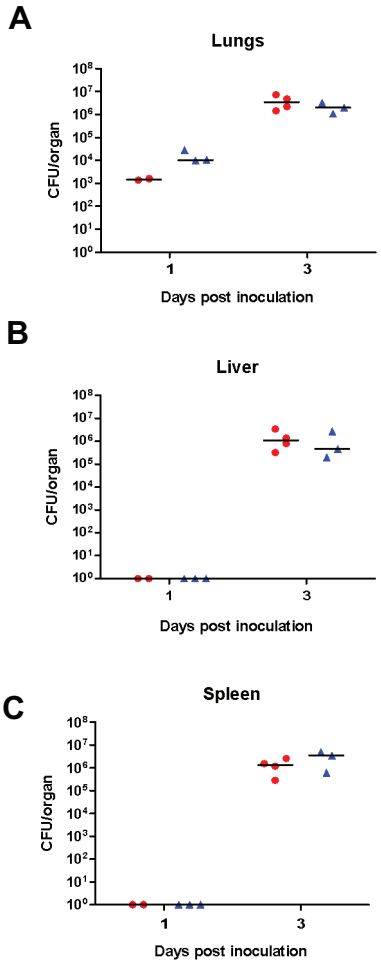


Figure 4

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

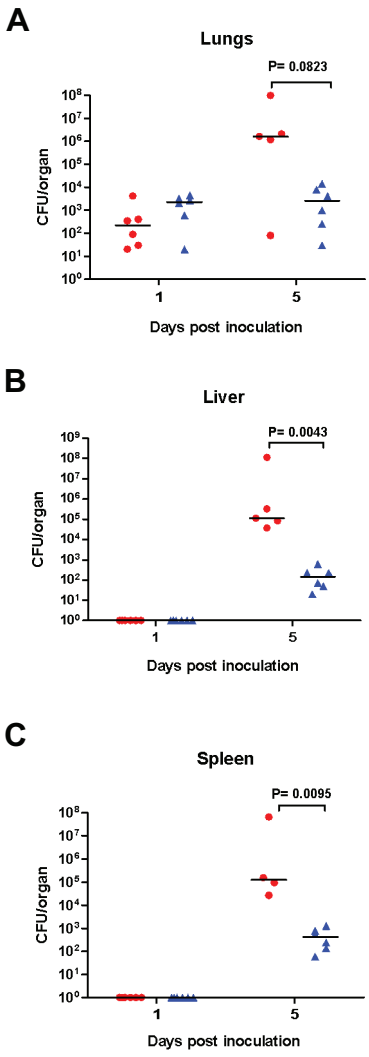


Figure 5

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

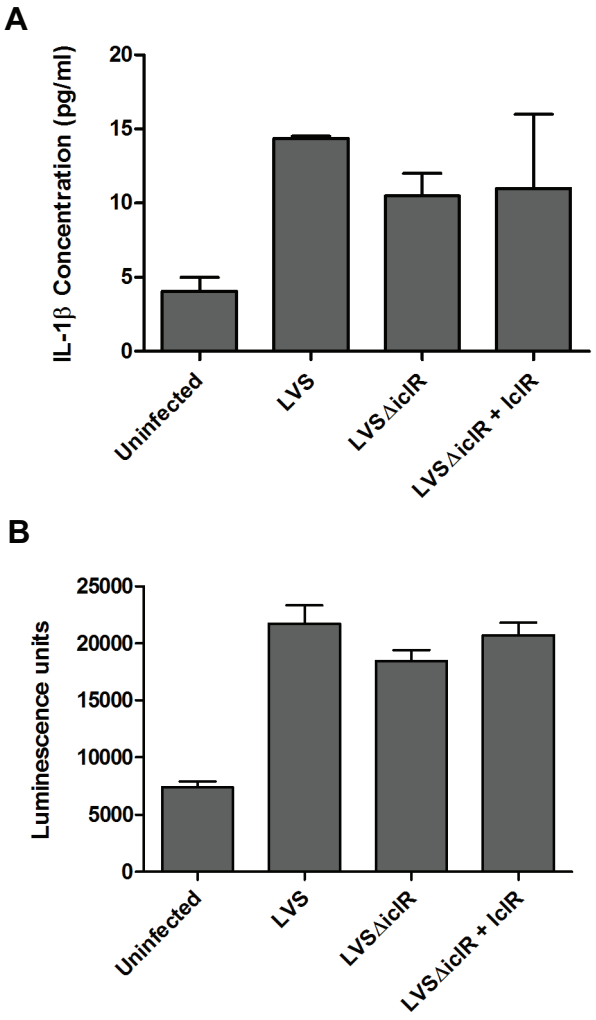


Figure 6

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

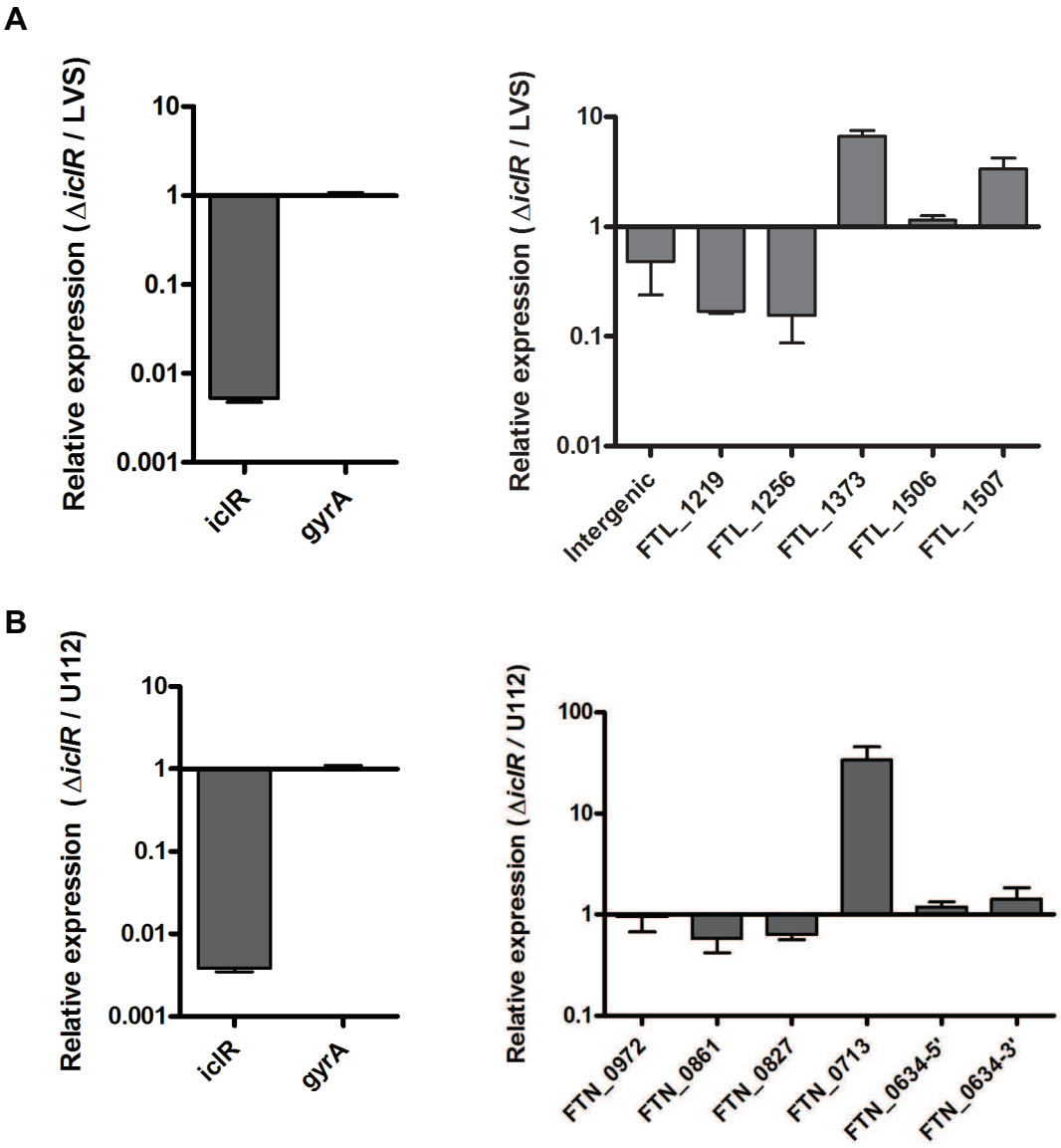


Figure 7

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest

IAI Accepts published online ahead of print

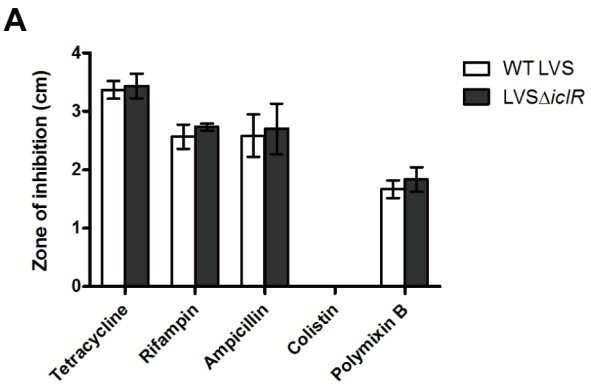


Figure 8

Downloaded from http://iai.asm.org/ on October 19, 2019 by guest