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Deletion of the MFS transporter *fptB* alters host cell interactions and attenuates the virulence of Type A *Francisella tularensis*

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Running Headline: Attenuation of *F. tularensis* by transporter deletion

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Abstract

Francisella tularensis is a Gram negative facultative intracellular coccobacillus that can infect a wide variety of hosts. In humans, *F. tularensis* causes the zoonosis tularemia following insect bites, ingestion, inhalation, and the handling of infected animals. That a very small inoculum delivered by the aerosol route can cause severe disease, coupled with the possibility of its use as an aerosolized bioweapon, have led to the classification of *Francisella tularensis* as a Category A select agent and has renewed interest in the formulation of a vaccine. To this end, we engineered a Type A strain SchuS4 derivative containing a targeted deletion of major facilitator superfamily (MFS) transporter *fptB*. Based on the attenuating capacity of this deletion in the *F. tularensis* LVS background, we hypothesized that deletion of this transporter would alter intracellular replication and cytokine induction of the Type A strain and attenuate virulence in the stringent C57BL/6J mouse model. Here we demonstrate that deletion of *fptB* significantly alters the intracellular lifecycle of *F. tularensis*, attenuating intracellular replication in both cell line and primary macrophages, and inducing a novel cytosolic escape delay. Additionally, we observed prominent differences in the *in vitro* cytokine profile in human macrophage-like cells. The mutant was highly attenuated in the C57BL/6J mouse model, and provided partial protection against virulent Type A *F. tularensis* challenge. These results indicate a fundamental necessity for this nutrient transporter in the timely progression of *F. tularensis* through its replication cycle, and in pathogenesis.

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Introduction

Francisella tularensis is a Gram negative facultative intracellular coccobacillus that is the causative agent of the zoonosis tularemia (1, 2). Two subspecies of *Francisella*, *F. tularensis* subspecies *tularensis* (Type A), and subspecies *holarctica* (Type B), account for virtually all instances of disease in humans, though a third, subspecies *novicida*, is not infectious in man, but is often used as a laboratory model. While there is no documented case of human to human transmission, *F. tularensis* can be spread to humans via inhalation of infected aerosols, the bite of insect vectors, the ingestion of contaminated food or water, and the handling of infected animals (3). Cutaneous exposure, most often via an insect vector, accounts for much of the disease burden and can lead to a protracted, though rarely fatal infection (4, 5). Conversely, inhalation of as few as 10-15 bacteria of the virulent Type A strain can cause serious illness in humans with a mortality rate approaching 50% if left untreated (6, 7). Several countries had utilized *Francisella* as a biological weapon during the middle of the 20th century due to its high virulence, low inoculum, and ease of delivery by the aerosol route (8). For this reason, the CDC has designated *Francisella tularensis* subspecies *tularensis* as a Tier 1 Category A select agent and a high priority for countermeasure development.

Currently, there is no licensed vaccine against *F. tularensis*, but development efforts continue in light of the potential threat to public safety. Past development efforts have included component and killed whole cell, or "Foshay" vaccines, but these were met with limited success (9-12). To date, the live-attenuated vaccine strategy has shown the most promise. One candidate strain derived from *F. tularensis* subspecies *holarctica* by the Soviet Union through the 1940s and 1950s was transferred to the U.S. in 1956, further passaged and became known as "live vaccine strain" (LVS) (7). *F. tularensis* LVS was tested extensively in clinical trials and

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demonstrated the feasibility of the live vaccine approach in conferring at least partial protection against Type A challenge. Subsequently, multiple attenuated live vaccine candidates have been engineered utilizing the *F. tularensis* subspecies *novicida*, *F. tularensis* LVS and *F. tularensis* Type A backgrounds (6, 13-22). These new vaccine candidates have shown varying degrees of efficacy in animal models and some are being further developed for use in humans. An additional outcome of these studies has been the discovery of novel information about the pathogenic process of *F. tularensis*, including alterations to and impacts on the pathogenic lifecycle incurred by targeted deletion of various genes in the attenuation process.

A hallmark of *Francisella* infection is the ability to induce phagocytosis via several routes to gain entry to many cell types, especially macrophages, where it rapidly escapes the phagosome to replicate to high numbers in the cytosol (23-27). Because of the distinctive tetraacylated structure of its lipopolysaccharide lipid A component, *Francisella* is largely able to evade TLR4-mediated recognition (28). Rather, *Francisella* initiates signaling through TLR2, resulting in the production of pro-inflammatory cytokines (29, 30), and, in a manner dependent on guanylate binding proteins (GBPs), STING, and mitochondrial reactive oxygen species, activates the AIM2 inflammasome (31-34). The virulence of *Francisella* stems from its ability to multiply to high numbers in the cytosols of infected cells, especially macrophages (35). As a result, many attenuation attempts have targeted metabolic or nutrient acquisition genes (13, 15-17, 36-38). We demonstrated previously that deletions in three of eight *Francisella phagosomal transporter* (*fpt*) genes, encoding members of the Major Facilitator Superfamily of secondary transporters, in the *F. tularensis* LVS background, resulted in significant *in vitro* and *in vivo* phenotypes: intracellular replication of these strains in macrophages and hepatocytes was reduced and the mutants were attenuated in BALB/c mice. Furthermore, immunization of mice

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with strains *F. tularensis* LVSΔ*fptB*, LVSΔ*fptE*, or LVSΔ*fptG* protected against homologous lethal challenge (13). We hypothesized that engineering deletion mutations in one of the most promising of these genes, *fptB*, an isoleucine transporter (38), in the *F. tularensis* Type A background would result in a protective vaccine candidate strain. Here we report that an *F. tularensis* strain SchuS4 mutant lacking *fptB* demonstrates altered intracellular replication and cytokine release kinetic profiles in macrophages, and is attenuated in mice, indicating its essential role in the intracellular lifecycle and virulence of *F. tularensis*.

Results

Generation of *fpt* deletion Type A *Francisella tularensis* SchuS4 strain

An *F. tularensis* Type A strain with an unmarked deletion of *fptB* was constructed using allelic exchange technology as described previously (13). Deletion of the target gene and the generation of mutant strain *F. tularensis* SchuS4Δ*fptB* was confirmed by PCR using primers originating in both gene flanks, and intragenically (Fig. S1). The substrate for *fptB* has been reported to be isoleucine in both *F. tularensis* subspecies *holarctica* and subspecies *novicida* (38). Given the 99% identity between the Type A and Type B gene sequences, we first confirmed isoleucine as the substrate for *fptB* using Chamberlain's defined medium (CDM); *F. tularensis* SchuS4Δ*fptB* exhibited reduced growth compared to WT in CDM, but could be restored to WT levels by the addition of exogenous isoleucine (Fig. 1).

The mutant strain SchuS4Δ*fptB* is deficient in intracellular replication and cellular escape in macrophages

The virulence of *Francisella* is dependent, in part, on its ability to replicate to high numbers intracellularly, especially in macrophages (35). The replication kinetics of the *fpt*

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107 mutant strain was compared to that of WT *F. tularensis* SchuS4 initially in THP-1 human
108 macrophage-like cells and HepG2 human hepatic carcinoma cells. In order to observe only the
109 initial round of invasion and replication, a bactericidal concentration of gentamicin was
110 maintained in the culture media to prevent cell-to-cell spread. In these conditions, there were
111 significantly higher numbers of the mutant strain in THP-1 macrophages at 24 hours post-
112 infection compared to the WT strain ($p < 0.0001$) (Fig. 2 A). WT *F. tularensis* doubled 3 to 4.5
113 times in the first 24 hours, compared to 5-5.4 times for *F. tularensis* SchuS4Δ*fptB*, significantly
114 more than the WT strain ($p < 0.01$) (Fig. 2 C).

115 When cell-to-cell spread was allowed to proceed by removal of gentamicin from the
116 media post-invasion, *F. tularensis* SchuS4Δ*fptB* exhibited a significant ($p < .0001$) replication
117 defect as evidenced by reduced numbers of intracellular bacteria and significantly fewer
118 doublings ($p < 0.001$) through 24 hours (Fig. 2 B, D). Without gentamicin, *F. tularensis*
119 SchuS4Δ*fptB* doubled only 6.8 times compared to 8.7 for WT (Fig 2 D). These data suggest that
120 *F. tularensis* SchuS4Δ*fptB* has a reduced intracellular replication rate compared to WT and is
121 delayed in cellular escape. *Trans*-complementation of the *fptB* gene with an intact plasmid-
122 encoded gene copy restored WT replication kinetics for the mutant strain in THP-1 cells (Fig.
123 S2). Furthermore, addition of 3 mM isoleucine, the reported substrate for FptB (38), to the
124 culture media of THP-1 cells infected with *F. tularensis* SchuS4Δ*fptB* was able to rescue the
125 replication defect in a similar fashion to broth assays. The addition of exogenous isoleucine
126 resulted in equivalent numbers of intracellular bacteria for *F. tularensis* SchuS4Δ*fptB* and WT at
127 24 hpi (Fig. 3 A). Similarly, the calculated doubling rate for the mutant in the presence of
128 isoleucine was significantly higher than that of the mutant without isoleucine ($p < 0.05$), and
129 statistically equivalent to that of WT (Fig. 3 B).

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130 Finally, intracellular growth kinetics were tested in the human hepatic carcinoma cell line
131 HepG2. Previous work demonstrated that the LVSΔ*fptB* mutant exhibited a replication defect
132 compared to the parental LVS strain with fewer mutant bacteria present intracellularly at 24 and
133 48 hpi. In contrast, SchuS4Δ*fptB* did not exhibit growth defect or delayed cell escape in this cell
134 type (data not shown).

135 **_F. tularensis_ SchuS4Δ*fptB* induces delayed cell death in THP-1 cells**

136 To examine more closely the escape delay phenotype displayed by *F. tularensis*
137 SchuS4Δ*fptB*, invasion assays in THP-1 macrophages were carried out under cell spread-limiting
138 conditions with time points every 6 hours during 33 hours of infection. Positive doubling rates
139 were interpreted as an indication that replication was outpacing escape, and negative doubling
140 rates as escape outpacing replication. Supernatants were assayed for the release of lactate
141 dehydrogenase (LDH), an intracellular enzyme whose release is indicative of cell death (39, 40).
142 Supporting our previous findings in THP-1 cells, *F. tularensis* SchuS4Δ*fptB*, exhibited a
143 significant growth defect that was quantified as reduced intracellular bacterial numbers, evident
144 by 15 hpi ($p < 0.05$) (Fig. 4 A) and reduced doublings between 3 and 9 hpi (Fig. 4 B). *F.*
145 *tularensis* SchuS4Δ*fptB* continued to double for 12 hours beyond that of WT, exhibiting
146 increasing CFU numbers (Fig. 4 A) and positive doubling values (Fig. 4 B) through 27 hpi, 12
147 hours beyond that of WT. In fact, *F. tularensis* SchuS4Δ*fptB* reached a significantly higher peak
148 intracellular numbers than WT ($p < 0.0001$). The release of LDH from *F. tularensis*
149 SchuS4Δ*fptB*-infected cells was similarly and significantly ($p < .0001$) delayed compared to
150 WT-infected cells (Fig. 4 C). The release kinetics indicate a 12-hour delay in the onset of cell
151 death.

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152 To further assess the differences in cellular escape between *F. tularensis* SchuS4Δ*fptB*
153 and WT, bacteria were sampled from the supernatants of infected THP-1 cells cultured without
154 antibiotics, as well as from the intracellular compartment. As had been shown previously, there
155 were significantly fewer *F. tularensis* SchuS4Δ*fptB* than WT CFU by 15 hpi ($p < 0.0001$) (Fig. 5
156 A). By 15 hpi, significantly more WT *F. tularensis* are found in the supernatants of infected cells
157 ($p < 0.01$) than SchuS4Δ*fptB* (Fig. 5 B). This increase in extracellular WT *F. tularensis*
158 correlates with the significant increase in LDH release seen previously at the 15 hpi time point
159 (Fig. 4 C). These results support a role for *fptB* in intracellular replication and timely escape from
160 the cell.

161 **_F. tularensis_ SchuS4Δ*fptB* elicits a delayed early pro-inflammatory response in host cells**

162 The production of TLR2-dependent cytokines, and the activation of STING and the
163 AIM2 inflammasome are necessary for initiation of the host cytokine response to *F. tularensis*
164 infection (30, 41-43). The altered intracellular replication kinetics of *F. tularensis* SchuS4Δ*fptB*
165 in macrophages suggested that infection with the mutant strain would likely result in modified
166 interactions with innate immune components. The levels of secretion of two important early pro-
167 inflammatory cytokines, TNF-α and IL-1β, were measured in supernatants from the THP-1
168 infection samples from the above assay. The release of IL-1β was delayed in *F. tularensis*
169 SchuS4Δ*fptB*-infected cells until 27 hpi, 12 hours later than WT-infected cells began secreting
170 IL-1β at a rate above uninfected cells (Figure 6 A). At 27 hpi, IL-1β secretion from cells infected
171 with *F. tularensis* SchuS4Δ*fptB* reached levels equivalent to that of WT. The timing of the IL-1β
172 release for both the mutant and WT coincides with that of LDH release, demonstrating a
173 correlation between cell death and mature IL-1β release. Similarly, infection of THP-1 cells with
174 WT *F. tularensis* induced a rapid and robust induction of TNF-α. In contrast, *F. tularensis*

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175 SchuS4Δ*fptB* induced the release of significantly less TNF-α compared to WT-infected cells at
176 all time points measured ($p < 0.0001$), despite increasing numbers of intracellular bacteria (Fig. 6
177 B).

178 **_F. tularensis_ SchuS4Δ*fptB* is replication deficient in primary macrophages**

179 In order to bridge studies between the THP-1 cell line and *in vivo* experiments in mice,
180 the replication kinetics of the *fpt* mutant strains was also examined in human monocyte derived
181 macrophages (hMDMs) and bone marrow derived macrophages (BMDMs) from C57BL/6J
182 mice. We hypothesized that growth attenuation in primary cells would be predictive of
183 attenuation *in vivo*. Significantly fewer intracellular *F. tularensis* SchuS4Δ*fptB* mutant bacteria
184 ($p < 0.001$) were counted at 24 hpi compared to WT (Fig. 7 A) in hMDMs. Correspondingly, the
185 number of doublings for *F. tularensis* SchuS4Δ*fptB*, was significantly lower than that of WT
186 between 8-24 hpi (1.5 versus 4.2 respectively, $p < 0.05$) (Fig. 7 B). Similar trends were observed
187 in mouse BMDMs. *F. tularensis*SchuS4Δ*fptB* elicited significantly fewer intracellular CFU at 24
188 hpi (Fig 7 C) This corresponded to a significant reduction in the calculated doubling rate
189 between *F. tularensis* SchuS4Δ*fptB* and WT between 8-24 hpi (1.2 versus 6.1 respectively, $p <$
190 0.0001) (Fig. 7 D).

191 **SchuS4Δ*fptB* is highly attenuated in the C57BL/6J mouse model**

192 Altered intracellular replication kinetics and cytokine induction patterns suggested a role
193 for *fptB* in *Francisella* virulence. The C57BL/6J mouse model was used to assess pathogenic
194 defects of these mutant strains as WT Type A *F. tularensis* has an intranasal $LD_{50} < 10$ CFU in
195 this model (44). Groups of 4 mice were inoculated intranasally with 5×10^1 CFU of WT, or $5 \times$
196 10^1 to over 7×10^6 CFU of the mutant strains. WT-inoculated mice steadily lost weight over the

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course of 5 days before succumbing to infection on day 5, exhibiting overt signs of sickness beginning late on day 4 or early on day 5. In contrast, no mice inoculated with *F. tularensis* SchuS4Δ*fptB* up to the highest dose succumbed to infection (Table 1). No weight loss or clinical signs of sickness were evident in any *F. tularensis* SchuS4Δ*fptB* inoculated mice, demonstrating attenuation and an $LD_{50} > 7.6 \times 10^6$ CFU.

***F. tularensis* SchuS4Δ*fptB* vaccination protects against virulent *F. tularensis* SchuS4 challenge**

Given the high level of attenuation of SchuS4Δ*fptB*, we assessed the ability of this strain to protect against lethal challenge. Groups of 4 mice in two independent experiments were inoculated with a single dose of ~1×10^6 CFU *F. tularensis* SchuS4Δ*fptB* before subsequent WT challenge 28 days later with doses ranging from 7 CFU - 360 CFU. Ten of 26 vaccinated mice from two separate experiments survived challenge, as well as one unvaccinated control resulting in an efficacy value of 28% (Table 2). An increased delay in time to death was observed in a challenge dose dependent manner (Table 2). All protected mice showed no overt signs of illness with little to no loss of weight following challenge. These data suggest *F. tularensis* SchuS4Δ*fptB* has limited protective capacity, but demonstrates the first example of protection by an MFS transporter-targeted vaccine strain from Type A challenge.

Discussion

The formulation of a licensed, efficacious live attenuated vaccine against *Francisella tularensis* has remained an elusive goal, despite increased efforts since the anthrax bioterror attacks of 2001. Prior studies in humans with *F. tularensis* LVS in the 1960's demonstrated the proof of concept for the protective capacity of the live attenuated vaccine strategy, and has

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219 pointed the way forward for the engineering of live attenuated strains harboring targeted
220 deletions (6, 13, 14, 19, 45). Here we investigated the potential of one MFS transporter FptB, as
221 an attenuating target in the virulent Type A strain, and the effects of its loss on the intracellular
222 lifecycle of Type A *F. tularensis*. Our prior studies demonstrated the importance of the *fptB* gene
223 in the *F. tularensis* LVS background, for both *in vitro* replication kinetics and *in vivo* virulence
224 (13). *F. tularensis* LVSΔ*fptB* exhibited a severe replication defect in multiple cell types and the
225 strain was both attenuated and protective in the BALB/c model (13). We hypothesized that,
226 given the high protein identity between the *F. tularensis* LVS and Type A strains for this protein
227 (99% for FptB), similar phenotypes would be observed in the clinically relevant Type A
228 background.

229 *F. tularensis* SchuS4Δ*fptB* displayed a significant growth defect in macrophages, both
230 cell-line and primary, similar to observations with the *F. tularensis* LVS-derived mutant, but in
231 contrast to our prior findings in LVS, did not harbor a replication defect in HepG2 cells (13, 38).
232 The restriction of intracellular growth defects to macrophages and not hepatic cells may stem
233 from the fact that the substrate of FptB, isoleucine, is regulated in macrophages. Isoleucine is one
234 of nine amino acids to be depleted by activated macrophages (46). This, coupled with the fact
235 that excess isoleucine in culture rescues growth of *F. tularensis* SchuS4Δ*fptB* implies that
236 isoleucine is a rate-limiting nutrient for intracellular replication of *Francisella tularensis*, and
237 that there likely exists another, previously hypothesized (38), lower affinity transporter that
238 contributes to transport of isoleucine in the absence of FptB. It may be that FptB functions as a
239 high affinity transporter to scavenge isoleucine intracellularly, even at depleted levels.

240 In addition to an intracellular replication defect, these data support an escape delay for
241 SchuS4Δ*fptB*. Multiple studies have demonstrated that *F. tularensis* does not replicate until it

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242 escapes from the phagosome (47-49). The positive intracellular replication rate of SchuS4Δ*fptB*,
243 measured as early as 3-9 hpi, demonstrates that the mutant strain has escaped from the
244 phagosome, similarly to WT, and is replicating in the cytosol, albeit at a reduced rate. However
245 the mutant continues to replicate intracellularly for an additional 12 hours beyond when WT *F.*
246 *tularensis* is primarily escaping. Further evidence demonstrating a delay in the emergence of *F.*
247 *tularensis* SchuS4Δ*fptB* in infected cell supernatants compared to WT, and a corresponding
248 delay in the onset of LDH release from mutant infected cells compared to WT-infected cells,
249 supports a delayed cellular escape phenotype. Specifically, at 21 hpi, *F. tularensis* SchuS4Δ*fptB*
250 and the WT strain are present at similar intracellular numbers, but only the WT strain is
251 triggering the release of LDH above background levels, suggesting that the WT strain is killing
252 cells, while the mutant is accumulating intracellularly. The delay in cytosolic escape observed
253 with the *fptB* mutant strain has not been reported previously in *Francisella*. Extensive work by
254 multiple laboratories has revealed the process by which *Francisella* escapes the phagosome early
255 in the replication cycle, what host and bacterial genes are key to this process, and the
256 consequences of delays at this stage (50-54). However, escape from the host cell at the final
257 stage of the intracellular cycle remains an aspect of the *Francisella* lifecycle that is poorly
258 understood. Current models of escape from macrophages involve Type A *Francisella* replicating
259 to high numbers before triggering cell death mediated through caspase 3 (55), during which the
260 bacteria are presumed to be released from the cell (35, 56, 57). While the escape process appears
261 tied to the cell death machinery, the control of timing of cell death and subsequent escape
262 includes both host and bacterial factors. Exactly what molecular steps define the escape path for
263 *F. tularensis* remain understudied. It has been demonstrated that *Francisella* triggers autophagy
264 in mouse embryonic fibroblasts and then associates with the autophagic machinery to harvest

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265 this microenvironment for nutrients, especially amino acids (58-61). The involvement of
266 autophagy in the *F. tularensis* life cycle and release in human cells remains largely unexplored,
267 but is an intriguing cell process to explore.

268 A fundamental key to the virulence of *Francisella* is its ability to invade and replicate
269 intracellularly in many cell types, especially macrophages, where host responses have been
270 extensively studied, revealing the induction of a potent, yet atypical inflammatory response that
271 can ultimately lead to sepsis and hypercytokinemia (62, 63). The avoidance of TLR4, yet
272 stimulation of TLR2/6, AIM2, and STING produces an immune response that can be less than
273 effective for host survival (28-30), and highlights the extreme virulence of this bacterium.
274 Infection with the *fpt* mutant strain elicited altered inflammatory cytokine secretion; *F. tularensis*
275 SchuS4Δ*fptB* infection resulted in a 12 hour delay in IL-1β secretion and a significantly reduced
276 TNF-α response across all time points measured. The level of attenuation in the C57BL/6J
277 mouse model was >700,000 times of that of WT for SchuS4Δ*fptB*. This high level of attenuation
278 exhibited by *F. tularensis* SchuS4Δ*fptB* places it among the most attenuated Type A-derived
279 vaccine strains (64), and confirms the importance of intracellular replication and host cell
280 interactions early in infection. Though we have not yet reached the lethal dose for this strain,
281 challenge results suggest that *F. tularensis* SchuS4Δ*fptB* may be over-attenuated. Historically,
282 the prime difficulty of live attenuated vaccine development has been achieving the optimal
283 balance of safety/attenuation and immunogenicity/protection. The reduced inflammatory
284 response elicited by *F. tularensis* SchuS4Δ*fptB* in the early stages of macrophage infection
285 compared to WT may indicate that an altered host cell interaction at the time of initial
286 macrophage infection does not guarantee the onset of a long-term protective response.

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These data represent the first utilization of an MFS transporter, an amino acid transporter specifically, as an attenuating target in the virulent *F. tularensis* Type A background. Prior efforts have centered upon biosynthetic (16, 18, 65), capsule (19, 66, 67), and virulence genes (68), but transporters represent a largely untapped source of potentially attenuating targets. Importantly, nutrient transporters, including those of the MFS, may be of increased importance to intracellular pathogens. It is appreciated that bacteria residing in the intracellular space undergo genome reductions, resulting in the shedding of genes involved in transport and metabolism at a rate that is much higher than that of genes involved in replication and defense (69). This implies that any remaining transporter and metabolite genes are necessary for the survival, and possibly, virulence of the organism. The Type A strain of *Francisella tularensis* is predicted to have 35 MFS transporters, and 182 transporters in total (70), representing close to 12% of the SchuS4 protein coding sequences. This represents a very large pool of potential novel targets for not just vaccines, but for antibiotic therapies as well. Intriguingly, similar gene proportions exist in many clinically relevant pathogenic organisms.

F. tularensis SchuS4Δ*fptB* is the first MFS-based live attenuated strain in the Type A background to demonstrate protection against WT Type A *Francisella* challenge. While the efficacy was relatively modest following vaccination with this strain, these data support the idea of targeting nutrient transporters as part of an attenuation strategy. These strains represent insightful tools allowing more intimate study the intracellular lifecycle of *F. tularensis*, especially the understudied molecular steps leading to cellular escape.

Materials and Methods

Bacteria and growth conditions. Bacterial strains utilized in this study are listed in Table S1 in the supplemental materials section. *Francisella tularensis* SchuS4 (BEI, Manassas, VA) was

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preserved at -80° C in Mueller-Hinton broth (MHB) (BD Microbiology Systems, Sparks, MD) with 15% glycerol added. Complete MHB includes with 1% Isovitalex (BD, Cockeysville, MD), 0.1% glucose, and 0.25% ferric pyrophosphate and was used for all liquid cultures. Mueller-Hinton agar (MHA) (BD Microbiology Systems, Sparks, MD) was used for solid cultures and was augmented as defined above, but also contained 10% defibrinated sheep blood (Lampire Biological Laboratories, Pipersville, PA). When selection for electroporants was being undertaken, kanamycin (Km) was added to MHA to a final concentration of 10 μg/mL. Suicide plasmids used in this study (Table S1) were preserved in *Escherichia coli* DH5α and grown in Luria-Bertani (LB) broth (BD Microbiology Systems, Sparks, MD) supplemented with 50 μg/mL kanamycin.

Deletion of *F. tularensis* genes *F. tularensis* was transformed via electroporation as previously described (13). Briefly, *F. tularensis* was first grown on MHA at 37° C in 5% CO_2 for two days, then was re-suspended in 1 mL of 0.5 M sucrose. Bacteria were pelleted and washed three times in 0.5 M sucrose before resuspending in a final volume of 300 μL. To this, 150 μL of suicide plasmid (13) prepared from 500 mL LB broth cultures (utilizing a Qiagen (Germantown, MD) Midi-Prep kit) was added. Electroporation was performed at 1.75 kV, 25 μF, and 600 Ω. After electroporation, cells were allowed to recover in MHB for 2 hours at 37° C in 5% CO_2 and then were plated onto kanamycin-containing MHA plates to select for cointegrants. Cointegration of the plasmid was confirmed using PCR. Plasmid curing was accomplished by growth in MHB containing 10% sucrose. These cultures were plated to MHA supplemented with 8% sucrose, and colonies arising here were screened by PCR to confirm loss of the targeted gene and plasmid. Primers utilized in screening are found in Table S2.

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Growth curves Growth curves comparing WT *F. tularensis* and *fpt* mutant strains were performed in Chamberlain's Defined Medium (CDM) (Teknova, Hollister, CA), or CDM supplemented with 3 mM isoleucine. Bacteria were grown on MHA plates and resuspended to a starting optical density at 600 nm (OD_{600}) of 0.1 in liquid media. Cultures were incubated with shaking at 37° C in 5% CO_2 for 7.5 hours, with OD_{600} readings taken every 1.5 hours.

Intracellular survival assays. The ability of *fpt* mutant strains to survive and replicate intracellularly was evaluated in the human THP-1 macrophage cell line (ATCC, Manassas, VA), the human HepG2 hepatic carcinoma cell line (ATCC, Manassas, VA), human monocyte derived macrophages (hMDM), and bone marrow derived (BMDM) macrophages from C57BL/6J mice.

THP-1 cells were cultivated in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 0.1% 1000X 2-mercaptoethanol (Gibco, Gaithersburg, MD), and were maintained at 37° C in 5% CO_2. Three days prior to the beginning of an assay, THP-1 cells were differentiated using phorbol myristate acetate (PMA) (Sigma Aldrich) at a concentration of 50 ng/mL for 24 hours. Media was then changed to supplemented RPMI 1640 as described above. For assays to assess bacterial replication, cells were infected at a multiplicity of infection (MOI) of 100 with either *F. tularensis* SchuS4 or *fpt* mutant for 2 hours. Following the 2 hour infection, cells were washed twice with PBS and incubated in RPMI 1640 containing 50 μg/mL gentamicin (Gibco, Gaithersburg, MD) for 1 hour. At 3 hours post infection (hpi), cells were washed again 2X with PBS, and placed into fresh RPMI 1640 lacking gentamicin. Intracellular bacterial replication was assayed at 3, 24, and 48 hours post-infection by lysing cells with a 0.02% SDS in PBS solution, followed by serial dilution and plating of the bacteria onto MHA. Culture

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supernatants were saved for downstream analyses. Bacterial doublings were calculated utilizing the following formula:

$$(\log_{10}T_n - \log_{10}T_{(n-1)}) \times 3.32$$

T_n is the number of colony forming units (CFU) at one time point, and $T_{(n-1)}$ is the CFU number at the prior time point. For assays that assess bacterial escape kinetics, the infection and washing steps were repeated as shown above through 3 hpi. Gentamicin-containing media was not washed off cells, and thus, cells were left for the duration of the assay in 50 μg/mL gentamicin. CFU were sampled as described above at the time courses indicated in the figures.

To assess the appearance of bacteria in the supernatants, THP-1 cells were infected and washed as described above. Gentamicin-containing media was removed at 3 hpi, and cells were placed in antibiotics-free media until CFU enumeration at 3, 9, 15, and 21 hpi. Supernatants were removed from the wells at the times specified above, and were serially diluted and plated on MHA. Intracellular bacteria were then enumerated as described above.

HepG2 cells were cultivated in minimal essential medium (MEM) supplemented with 10% FBS and maintained at 37° C in 5% CO_2. To assess the intracellular survival abilities of *fpt* mutant strains compared to that of WT *F. tularensis*, HepG2 cells were also seeded at a density of 1×10^6 cells per well in 12-well plates and infected with either *F. tularensis* SchuS4 or *fpt* mutant strains at an MOI of 300 for 2 h. After the 2 hour infection period, cells were washed twice with PBS and then incubated in MEM containing 50 μg/ml gentamicin for 1 hour. Next, cells were either left in gentamicin-containing media for the duration of the experiment, or were washed twice with PBS and then incubated in antibiotic-free MEM for the duration of the experiment. Bacterial replication was assayed at 3, 24, and 48 hours post-infection by lysing the cells with 0.02% SDS/PBS and plating the bacteria onto MHA.

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377 **Supplemented intracellular survival assays.** The ability of isoleucine supplementation to
378 rescue intracellular replication of *F. tularensis* SchuS4Δ*fptB* was examined in THP-1
379 macrophage-like cells. THP-1 cells were prepared and infected as described above. Immediately
380 before the start of the assay, all cells were washed and placed in either standard RPMI 1640, or
381 RPMI 1640 supplemented with 3 mM isoleucine. Such media conditions were maintained
382 throughout the entirety of the assay before CFU enumeration at 24 hours post-infection.
383 **Isolation and culture of mouse bone marrow derived macrophages** Femurs were extracted
384 from 6-8 week old C57BL/6J mice (University of Maryland, Baltimore Veterinary Resources
385 Breeding Facility) and flushed with Dulbecco's Minimal Essential Medium (DMEM)
386 supplemented with 10% low endotoxin FBS (Gemini Bioproducts, West Sacramento, CA), 30%
387 L929 cell supernatants (as a source of CSF-1), 1% non-essential amino acids (ThermoFisher
388 Scientific, Waltham, MA), 1% HEPES (ThermoFisher Scientific, Waltham, MA), and 1%
389 penicillin/streptomycin (ThermoFisher Scientific, Waltham, MA). Bone marrow cells were
390 passed through a 70 μm nylon mesh (Fisher Scientific, Waltham, MA) to remove debris and
391 were placed into a T175 (Costar, Corning, NY) flask for culture and differentiation. Fresh media
392 was placed on the day after extraction, and every other day subsequently for at least 1 week. The
393 day before an assay, cells were scraped, spun down at 1,100 RPM (125 × g) for 10 minutes, and
394 resuspended at a concentration of 5×10^5 per mL in media lacking penicillin/streptomycin. Cells
395 were plated at a concentration of 5×10^5 per well in a 24 well plate (Costar, Corning NY).

396 **Isolation and culture of human monocyte derived macrophages** Monocyte derived
397 macrophages (MDMs) were isolated from 100 mL whole human blood gathered in EDTA tubes
398 (BD Microbiology Systems, Sparks, MD) by the University of Maryland, Baltimore Center for
399 Vaccine Development clinical staff. Blood was diluted 1:2 in PBS before being added to 15 mL

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of ficoll (GE Healthcare, Laurel, MD) in SepMate tubes (Stem Cell Technologies, Cambridge, MA). SepMate tubes were spun at 1,200 xg for 15 minutes at room temperature. Red blood cells were depleted using ACK lysis buffer (Gibco, Gaithersburg, MD). Isolated MDMs were cultured for one week before the start of an assay in T75 flasks (Costar, Corning, NY) with RPMI 1640 supplemented with 10% low endotoxin FBS (Gemini Scientific, West Sacramento, CA), 1% non-essential amino acids (ThermoFisher Scientific, Waltham, MA), 1% HEPES (ThermoFisher Scientific, Waltham, MA), and 1% sodium bicarbonate (ThermoFisher Scientific, Waltham, MA). Media was changed daily. Two days before the start of an assay, cells were scraped from the flask, enumerated, and plated at a density of 5 x 10^5 cells per well in a 24 well plate (Costar, Corning, NY). These studies were approved by the University of Maryland, Baltimore Institutional Review Board.

Complementation of mutants A plasmid containing *fptB* under the control of the *F. tularensis guaB* promoter, named p*FT906-fptB* (13), was electroporated into the *F. tularensis* SchuS4 mutant strain as described previously. The complemented mutant wwas then tested via invasion assay in THP-1 macrophage-like cells for reversion to WT growth mechanics.

Intracellular replication time course assay. The timing of host cellular escape of *fpt* mutant strains compared to that of WT *F. tularensis* SchuS4 was evaluated in THP-1 macrophage-like cells. Differentiated THP-1 cells were seeded at a density of 1×10^6 then infected at an MOI of 100 with either *F. tularensis* SchuS4 or the *fpt* mutant strain for 2 hours. Following the 2 hour infection, cells were washed twice with PBS and then incubated in RPMI 1640 containing 50 µg/ml gentamicin for the remainder of the assay. Supernatants were collected for subsequent measurement of lactate dehydrogenase (LDH) and cytokine release, and CFU were determined

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422 by lysing the cells with a 0.02% SDS solution in PBS followed, by serial dilution and plating of
423 the bacteria onto MHA at 3, 9, 15, 21, 27, and 33 hours post-infection.

424 **Lactate dehydrogenase release assay.** LDH release was measured using a Cytotox 96
425 (Promega, Madison, WI) kit according to the manufacturer's protocol. Measurement of samples
426 was done using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA) at 490 nm
427 wavelength.

428 **Cytokine ELISAs** To assay secretion of interleukin-1β (IL-1β) and tumor necrosis factor-α
429 (TNF-α) from THP-1 cells, ELISA kits from R&D Systems (Minneapolis, MI). Supernatants
430 collected at 3, 9, 15, 21, 27, and 33 hours post-infection were assayed per the manufacturer's
431 protocol, and were read using a VersaMax plate reader at 450 nm with correction set at 540 nm.

432 **Mouse survival studies.** Six-8 week old male and female C57BL/6J were housed in the
433 University of Maryland Animal Biohazard Safety Level 3 (ABSL-3) facility for the duration of
434 the studies. All experiments were performed according to protocols approved by the UMB
435 Institutional Animal Care and Use Committee. To ascertain whether the *fpt* mutant strain was
436 attenuated, a mouse infection model was used. Groups of 4 C57BL/6J mice per dosage
437 concentration (2 males 2 females) were anesthetized, and were inoculated intranasally with either
438 WT *F. tularensis* SchuS4 or the *fpt* mutant suspended in 20 µL of PBS. Mice were monitored
439 daily for survival and clinical signs of infection (weight loss, lethargy, ruffling of fur) for 14 days
440 post-infection.

441 Mouse health was scored based on the following criteria: Condition 1 – normal activity;
442 mice run freely and energetically around the cage and resist when picked up. Condition 2 – mice
443 are slower than usual and offer less resistance when picked up. Condition 3 – mice exhibit a

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444 hunched posture, move very slowly, and display ruffled, dull fur and squinted eyes. Condition 4
445 – mice are almost entirely sedentary, hunched and are either unresponsive or barely responsive to
446 prodding. Fur is very ruffled and dull. Mice reaching a clinical score of 4 or losing > 20%
447 bodyweight were euthanized as required by the UMB IACUC.

448 **Mouse challenge studies.** Six-8 week old male and female C57BL/6J were immunized as
449 described above using a single vaccination regimen. Mice received ~1 × 10^6 CFU of *F. tularensis*
450 SchuS4Δ*fptB* and were challenged with WT *F. tularensis* SchuS4 intranasally 4 weeks later with
451 the doses outlined in Table 2. After challenge, all mice were monitored daily to check for clinical
452 signs and weight loss as described above. Delay to death for vaccinated, challenged mice was
453 calculated using the average day of death post challenge of the unvaccinated control group and
454 the average day of death for vaccinated mice that succumbed to challenge. The average day of
455 death for each group was calculated, and the average day of death for the control group was
456 subtracted, giving the delay to death per group. Mice that survived challenge were excluded from
457 these calculations.

458 **Statistical Analysis.** Using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA), two-way
459 analysis of variance (ANOVA) with Tukey's post-test was performed to assess statistical
460 significance between log-transformed bacterial CFU counts, LDH release levels, and cytokine
461 ELISAs ($p < 0.05$). A two-sided t test was utilized to determine significance between calculated
462 bacterial doubling values ($p < 0.05$). Calculated *p*-values for the t tests were adjusted using the
463 Benjamini, Krieger, and Yekutieli method with a false discovery rate of 1%. In all instances, WT
464 *F. tularensis* SchuS4 served as the reference strain for all statistical tests.

465 ## Acknowledgements

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Table 1: Attenuation of *fptB* deletion strain in C57BL/6J mice.

686

Strain	Intranasal Dose (CFU)	Survival
SchuS4	1.10×10^1	0/2
SchuS4	1.90×10^1	0/4
SchuS4	4.30×10^1	0/4
SchuS4	5.30×10^1	0/4
SchuS4Δ*fptB*	5.40×10^1	3/3
SchuS4Δ*fptB*	3.10×10^2	4/4
SchuS4Δ*fptB*	5.60×10^2	3/3
SchuS4Δ*fptB*	1.01×10^3	4/4
SchuS4Δ*fptB*	8.70×10^3	4/4
SchuS4Δ*fptB*	9.80×10^4	4/4
SchuS4Δ*fptB*	1.02×10^5	2/2
SchuS4Δ*fptB*	1.06×10^6	4/4
SchuS4Δ*fptB*	7.60×10^6	3/3

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688 **Table 2:** Protection against wildtype challenge after vaccination with SchuS4Δ*fptB*

Vaccine Inoculum (CFU)	Challenge Dose SchuS4 (CFU)	Survival	Individual Delay to Death (Days)	Average Delay to Death (Days)
7.30×10^5	7	2/5	8, 9, 8, 14<, 14<	4
7.30×10^5	70	1/4	7, 6, 6, 14<	2
7.30×10^5	162	2/5	6, 6, 6, 14<	1.67
PBS	350	0/3	4, 4, 5	--
1.14×10^6	39	2/4	7, 10, 14<, 14<	3.5
1.14×10^6	78	1/4	6, 7, 9, 14<	2.33
1.14×10^6	360	2/4	7, 6, 14<, 14<	1.5
PBS	39	1/4	5, 5, 5, 14<	--

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690 **Figure legends**

691 **Figure 1:** Growth in Chamberlain's Defined Medium. Growth kinetics of WT *F. tularensis*
692 SchuS4 and SchuS4Δ*fptB* were examined in Chamberlain's defined medium with or without
693 supplementation with 3 mM isoleucine. Data are of a single representative experiment (n = 3).

694 **Figure 2:** *F. tularensis* SchuS4Δ*fptB* exhibits altered intracellular replication kinetics in THP-1
695 cells. THP-1 cells were infected at an MOI of 100 for 2 hours with *F. tularensis* WT or
696 SchuS4Δ*fptB*. Cells were then washed twice with PBS and incubated with 50 µg/mL gentamicin
697 for either 1 hour followed by washing and incubation without antibiotics (B, D), or for the
698 duration of the experiment (A, C). Intracellular bacteria were enumerated at 3, 24, and 48 hpi and
699 number of doublings calculated between each interval. Data are presented as mean ± SEM with
700 three biological replicates for each experiment. Data are of a single representative experiment (n
701 = 5). ** $p < 0.01$, *** $p < 0.001$, ****, $p < 0.0001$ (Average CFU analyzed by two-way
702 ANOVA and calculated doublings analyzed by two-sided t test with Benjamini, Krieger, and
703 Yekutieli *p* value adjustment).

704 **Figure 3:** Excess isoleucine restores *F. tularensis* SchuS4Δ*fptB* replication kinetics to WT levels
705 in THP-1 cells. THP-1 cells were infected at an MOI of 100 for 2 hours. Cells were then washed
706 twice with PBS and incubated with 50 µg/mL gentamicin for 1 hour before being returned to
707 media lacking gentamicin. Intracellular bacteria were enumerated at 3, 24, and 48 hours post-
708 infection. Data are presented as mean ± SEM with three biological replicates for each
709 experiment. Data are of a single representative experiment (n = 2). *, $p < 0.05$, **, $p < 0.01$,
710 ****, $p < 0.0001$(Average CFU analyzed by two-way ANOVA and calculated doublings
711 analyzed by two-sided t test with Benjamini, Krieger, and Yekutieli *p* value adjustment).

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712 **Figure 4:** *F. tularensis* SchuS4Δ*fptB* induces a delay in cell death and LDH release. THP-1 cells
713 were infected at an MOI of 100 for 2 hours with *F. tularensis* WT or SchuS4Δ*fptB*. Cells were
714 then washed twice with PBS and incubated with 50 µg/mL gentamicin for the duration of the
715 experiment. Intracellular bacteria were enumerated at 3, 9, 15, 21, 27 and 33 hpi, doublings
716 calculated between each time interval, and supernatants were analyzed for LDH release. Data are
717 presented as mean ± SEM with three biological replicates for each experiment. Data are of a
718 single representative experiment (n = 4) *, $p < 0.05$, ** $p < 0.01$, ****, $p < 0.0001$ (Average
719 CFU analyzed by two-way ANOVA and calculated doublings analyzed by two-sided t test with
720 Benjamini, Krieger, and Yekutieli *p* value adjustment).

721 **Figure 5:** *F. tularensis* SchuS4Δ*fptB* is delayed in release to infected cell supernatants. THP-1
722 cells were infected at an MOI of 100 for 2 hours with *F. tularensis* WT or SchuS4Δ*fptB*. Cells
723 were then washed twice with PBS and incubated with 50 µg/mL gentamicin for one hour, before
724 being washed twice more with PBS and being returned to media lacking gentamicin. Intracellular
725 and extracellular bacteria were enumerated at 3, 9, 15, and 21 hpi. Data are presented as mean ±
726 SEM with three biological replicates for each experiment. Data are of a single representative
727 experiment (n = 2) ** $p < 0.01$, ****, $p < 0.0001$ (Average CFU analyzed by two-way ANOVA
728 and calculated doublings analyzed by two-sided t test with Benjamini, Krieger, and Yekutieli *p*
729 value adjustment).

730 **Figure 6:** *F. tularensis* SchuS4Δ*fptB* elicits altered inflammatory responses. THP-1 cells were
731 infected at an MOI of 100 for 2 hours. Cells were then washed twice with PBS and incubated
732 with 50 µg/mL gentamicin for the duration of the experiment. Cell-free supernatants were
733 collected at 3, 9, 15, 21, 27 and 33 hpi and measured for cytokine contents using sandwich
734 ELISAs. Data are presented as mean ± SEM with three biological replicates for each experiment.

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Data are of a single representative experiment (n = 5). ** $p < 0.01$, ****, $p < 0.0001$ (by two-way ANOVA).

Figure 7: *F. tularensis* SchuS4Δ*fptB* is deficient in replication in primary cells. . Human MDM cells (A, B) and mouse BMDM cells (C, D) were infected at an MOI of 10 for 2 hours. Cells were then washed twice with PBS and incubated with 50 µg/mL gentamicin for 1 hour before being returned to media lacking gentamicin. Intracellular bacteria were enumerated at 3, 8, and 24 hours post-infection. Data are presented as mean ± SEM with three biological replicates for each experiment. Data are of a single representative experiment (n = 4). *, $p < 0.05$, ****, $p < 0.0001$ (Average CFU analyzed by two-way ANOVA and calculated doublings analyzed by two-sided t test with Benjamini, Krieger, and Yekutieli *p* value adjustment).

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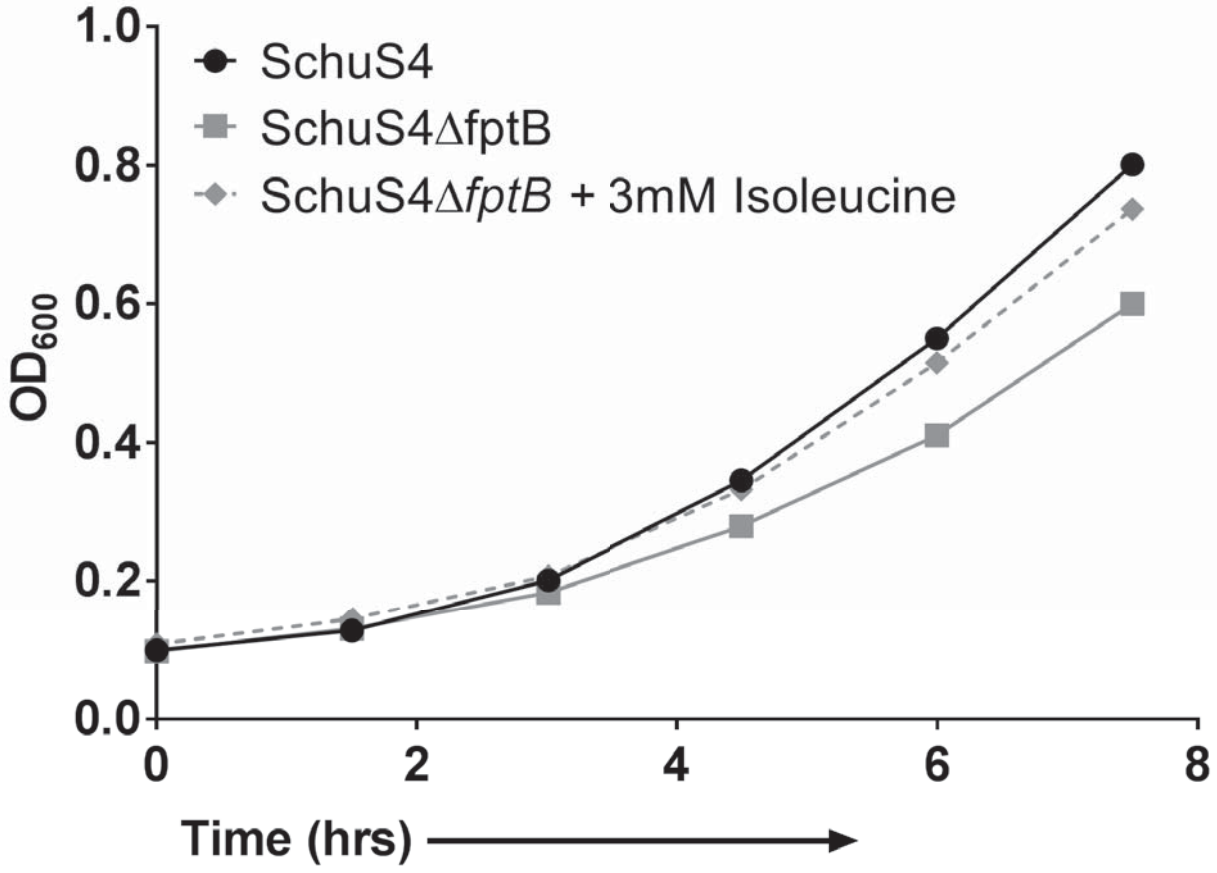

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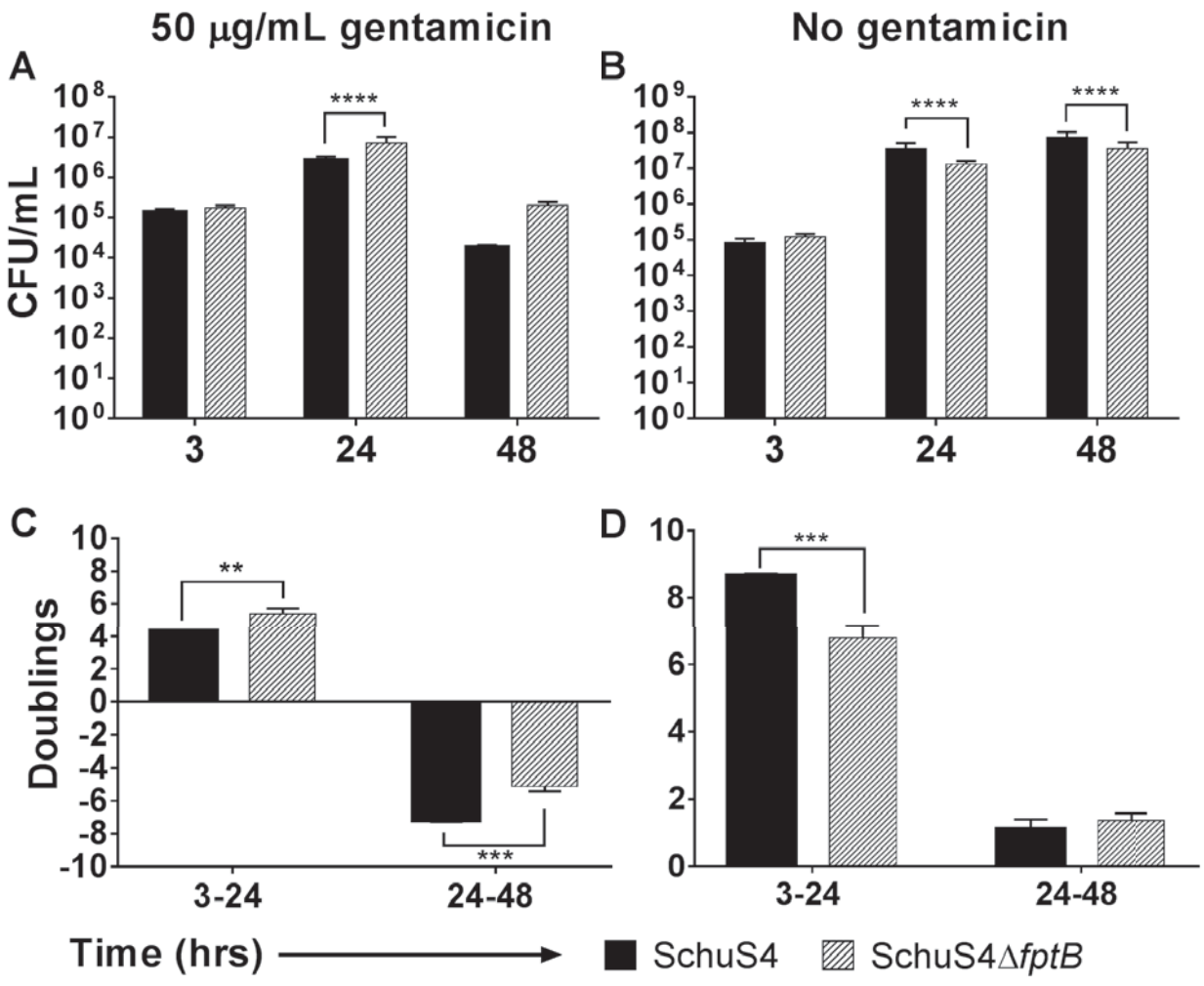

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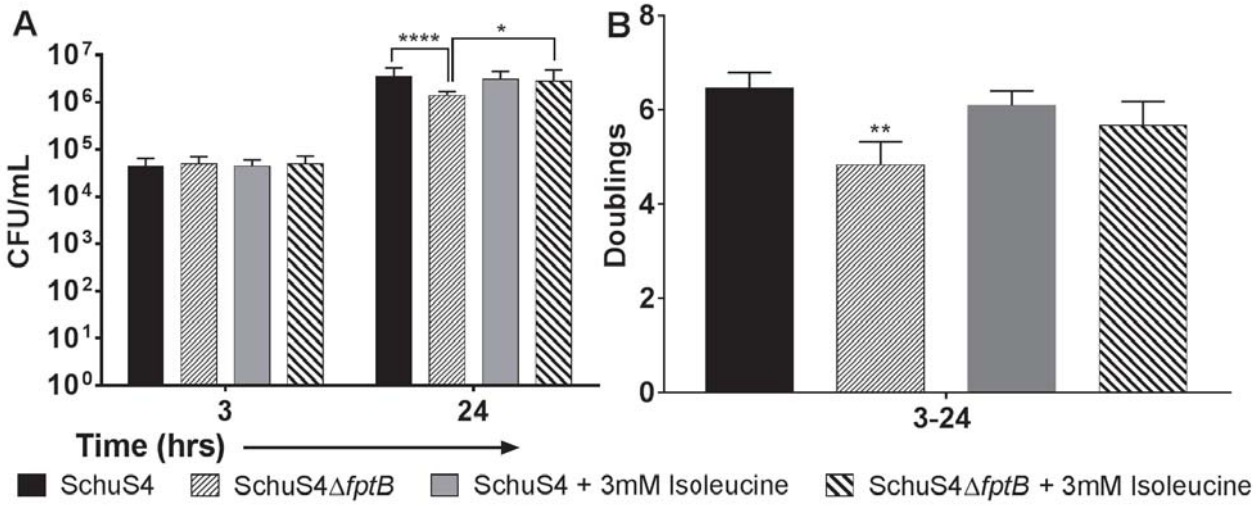

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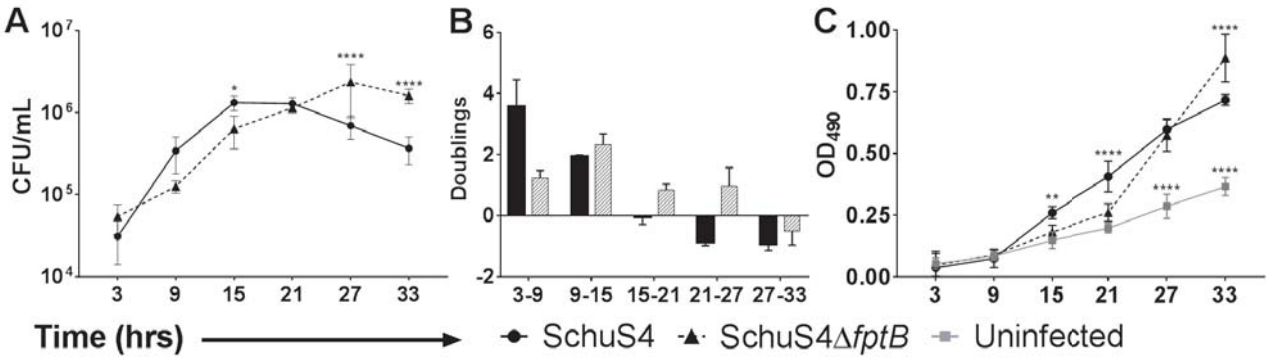

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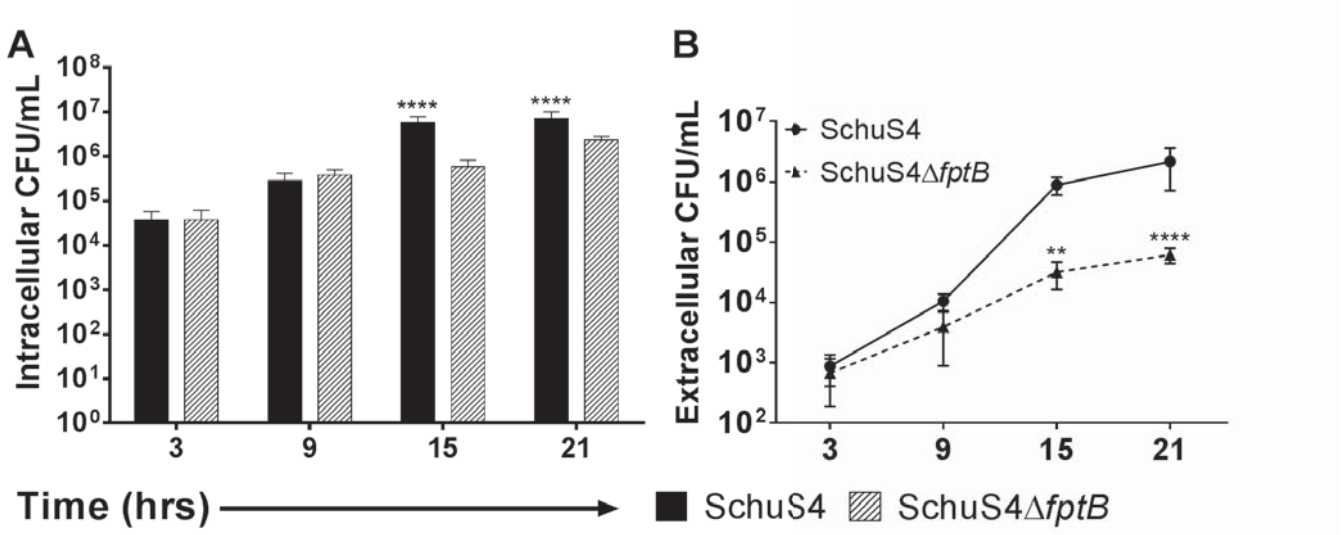

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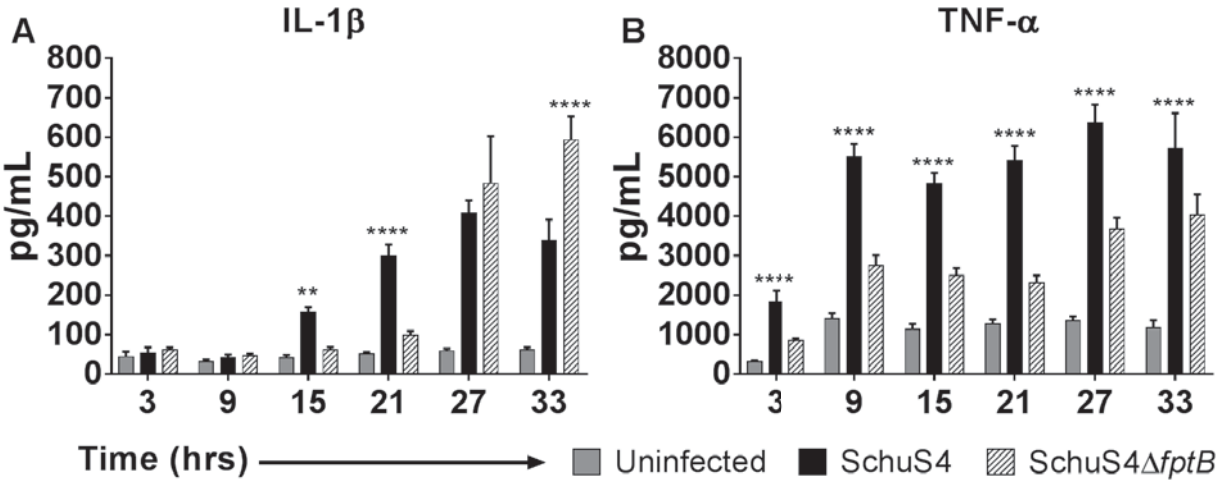

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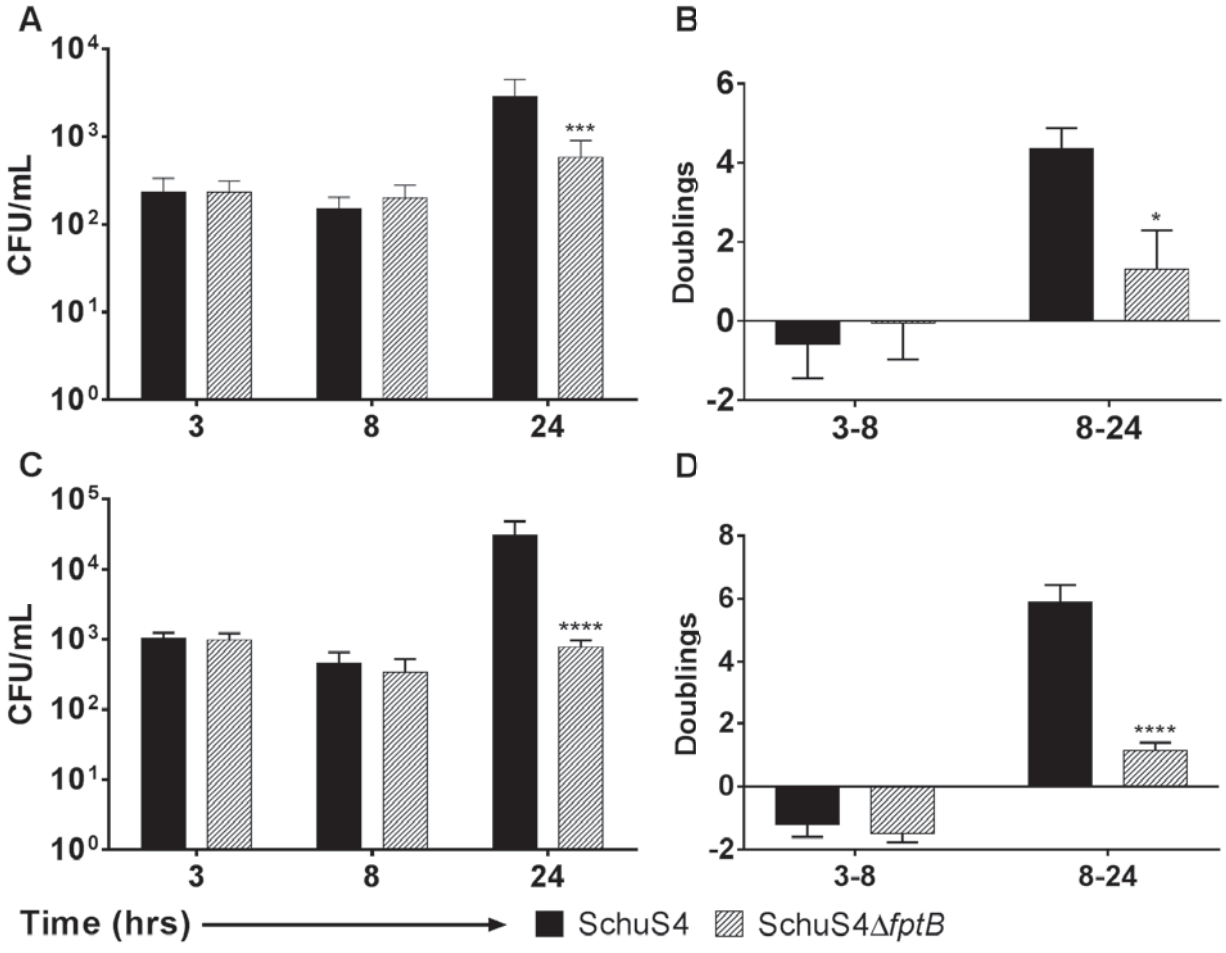

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