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1 A Single Mechanosensitive Channel Protects *Francisella tularensis* subsp. *holarctica* from
2 Hypoosmotic Shock and Promotes Survival in the Aquatic Environment.
3
4
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21 **Abstract**

22 *Francisella tularensis* subspecies *holarctica* is found throughout the northern hemisphere and
23 causes the disease tularemia in humans and animals. An aquatic cycle has been described for this
24 subspecies, which has caused water-borne outbreaks of tularemia in at least 10 countries. In this
25 study, we sought to identify mechanosensitive channel(s) required for the bacterium to survive
26 the transition from mammalian hosts to freshwater, which is likely essential for transmission of
27 the bacterium between susceptible hosts. A single mechanosensitive channel MscS (FTL_1753),
28 among the smallest members of the mechanosensitive channel superfamily, was found to protect
29 subsp. *holarctia* from hypoosmotic shock. Deletion of this channel did not affect virulence
30 within the mammalian host, however *mscS* was required to survive the transition from the host
31 niche to fresh water. Deletion of *mscS* did not alter the sensitivity of *F. tularensis* subspecies
32 *holarctica* to detergents, H_2O_2, or antibiotics, suggesting that the role of MscS is specific to
33 protection from hypoosmotic shock. Interestingly, deletion of *mscS* also led to reduced average
34 cell size without altering gross cell morphology. The small mechanosensitive channel identified
35 and characterized in this study likely contributes to the transmission of tularemia between hosts
36 by allowing the bacterium to survive the transition from mammalian hosts to fresh water.

37

38 **Importance**

39 Contamination of fresh water by *Francisella tularensis* subspecies *holarctica* has resulted in a
40 number of outbreaks of tularemia. Invariably, contamination originates from the carcasses or
41 excreta of infected animals, and thus involves an abrupt osmotic shock as the bacteria enter fresh
42 water. How *F. tularensis* survives this drastic change in osmolarity has not been clear, but here
43 we report that a single mechanosensitive channel protects the bacterium from osmotic

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44 downshock. This channel is functional despite being notably smaller (165 a.a.) than those found
45 in model organisms (~280 a.a.). These findings extend our understanding of the aquatic cycle
46 and ecological persistence *of F. tularensis*, with further implications for mechanosensitive
47 channel biology.
48

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49 **Introduction**

50 *Francisella tularensis* is a gram-negative bacterium responsible for the disease tularemia in
51 humans and a wide range of animal species. Two subspecies of *F. tularensis* are of clinical
52 significance for humans, ssp. *tularensis* and *holarctica* (1). Subspecies *tularensis* is considered
53 more virulent, and is primarily confined to Northern America. Subspecies *holarctica* on the other
54 hand is broadly distributed throughout the northern hemisphere, and has also been found in
55 Australia (2). The manifestations of tularemia vary depending upon the route of exposure. The
56 ulceroglandular and glandular forms of tularemia are more frequent and are associated with
57 exposure via arthropod bites or direct contact with infected animals, leading to localized
58 lymphadenopathy with or without the formation of an ulcer at the inoculation site (1, 3, 4).
59 Another common manifestation is oropharyngeal tularemia, where ingestion of contaminated
60 water leads to pharyngitis and swelling of the cervical lymph nodes (1). Rare presentations
61 include oculoglandular tularemia, involving conjunctivitis resulting from inoculation of the eye;
62 as well as typhoidal and pneumonic tularemia, which are both severe systemic diseases resulting
63 from inhalation of *F. tularensis* (pneumonic) or with no obvious route of exposure (typhoidal)
64 (3).
65
66 Tularemia is considered a zoonotic disease that is rarely, if ever, transmitted by human-to-human
67 contact (3). Hence, understanding how the bacterium persists and spreads in the environment is
68 important. While long-term reservoirs and transmission cycles of *F. tularensis* are not well
69 understood (5), the role of fresh water is of particular interest in the ecology of the broadly
70 distributed *F. tularensis* subsp. *holarctica*, for which an aquatic cycle has been described (1).
71 Many large outbreaks of tularemia are of the oropharyngeal form, and are linked to direct

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72 ingestion of water contaminated with *F. tularensis* subsp. *holarctica*. Outbreaks of tularemia
73 from contaminated drinking water have been reported in at least 10 countries, totaling thousands
74 of cases. Such outbreaks have been reported in Turkey (6–8), Sweden (9), Germany 10), Norway
75 11–13), Russia (14, 15), Bulgaria (16, 17), Kosovo (18, 19), The Czech Republic(20), Italy (21,
76 22) and the Republic of Georgia (23). Isolated cases of tularemia associated with infection from
77 fresh water have also been described in France (24, 25) and the USA (26). In many of these
78 cases, *F. tularensis* was detected by PCR (10–13, 20) or cultured (8, 9, 15–17, 23, 26) from
79 water of the affected area.
80
81 As the presence of viable *F. tularensis* subsp. *holarctica* in fresh water is directly responsible for
82 many naturally occurring cases of oropharyngeal tularemia, it is important to understand how the
83 bacterium adapts and survives in water. *F. tularensis* subsp. *holarctica* has been shown to
84 survive in otherwise sterile fresh water for 7-40 days (27, 28). Persistence may be enhanced by
85 the presence of amoebae or ciliates (29, 30), but the bacterium is not able to persist in fresh water
86 indefinitely (27, 28), and is often only seasonally recovered from natural bodies of water (7, 31).
87 This suggests that *F. tularensis* is introduced to bodies of water shortly before outbreaks, and
88 indeed almost all cases of water contamination are attributed to the carcasses or excreta of
89 infected animals including hares, rodents, voles, lemmings, muskrats, and beavers (6–8, 10–12,
90 16, 17, 19, 22, 26, 31–33). The shift from an animal to a fresh water niche involves an abrupt
91 decrease in extracellular osmolarity, producing a hypoosmotic shock (or 'downshock').
92
93 Downshock results in an rapid influx of water into the cytoplasm, increasing tension on the
94 membrane and potentially leading to lysis of the cell (34). To prevent this outcome, bacteria rely

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95 on mechanosensitive channels, which open in response to physical stretch of the membrane and
96 jettison osmolytes (35). By doing so, mechanosensitive channels relieve pressure, restore
97 isotonicity, and allow survival of hypoosmotic shock (36). In this study, we sought to identify the
98 mechanosensitive channel(s) that protect *F. tularensis* subsp. *holarctica* from hypoosmotic
99 shock, allowing it to survive the transition from a host to fresh water. We bioinformatically
100 identified prospective mechanosensitive channels in subsp. *holarctica*, deleted candidate genes,
101 and characterized the resulting mutants. We show that a single Mechanosensitive Ion Channel
102 protein (MscS) is required for surviving the transition from mammalian cells to fresh water.
103 Notably, the mechanosensitive channel gene identified herein is among the smallest members of
104 the mechanosensitive channel superfamily. Bacteria lacking this channel display a specific defect
105 in survival of osmotic shock, -while sensitivities to detergents, H_2O_2 and antibiotics were not
106 affected. We also demonstrate that deletion of this gene results in a decrease in cell size, but does
107 not affect the bacterium's ability to replicate in macrophages or cause disease in a mouse model
108 of tularemia. Together, these data indicate that MscS is critical for survival of *F. tularensis* in
109 fresh water environments, and plays a major role in the bacterium's ecological persistence.
110

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111 **Results**

112 ***F. tularensis Live Vaccine Strain mechanosensitive channels closely resemble those found in***
113 ***clinical and environmental strains.***

114 We first sought to compare putative mechanosensitive channels in various *F. tularensis* subsp.
115 *holarctica* strains, in order to determine whether the Live Vaccine Strain (LVS) was an accurate
116 representation of other members of the subspecies. We compared LVS to a human clinical
117 isolate (FSC200), an isolate from a beaver (OSU18) and an isolate from drinking water (R13-
118 38). Our search yielded four loci predicted to code for mechanosensitive channels in each
119 genome (FTL_1753, FTL_0945, FTL_1588, and FTL_1209 in LVS). Two genes (FTL_1753 and
120 FTL_0945) were identical in all four strains of subsp. *holarctica* (Fig. S1 & S2). When we
121 performed our initial bioinformatic comparison, FTL_1588 was annotated as a pseudogene in
122 RefSeq (8-12-2015 annotation with NCBI software revision 3.0). Nevertheless, our analysis of
123 FTL_1588 revealed that it was identical between LVS and FSC200 (Fig. S3). However, in
124 OSU18, a small deletion at the beginning of the open reading frame, a potential membrane
125 localization sequence, was observed (Fig. S3). A gap in the WGS assembly of R13-38 precluded
126 this strain from comparison. The final hit was annotated as a pseudogene (FTL_1209) due to
127 frameshift in all four strains. Taken together, these data show that the Live Vaccine Strain codes
128 for mechanosensitive channels and is an accurate representation of clinical and environmental
129 subsp. *holarctica* strains.

130

131 ***A single mechanosensitive channel protects F. tularensis subsp. holarctica from hypoosmotic***
132 ***shock.***

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133 We next sought to determine which of the predicted mechanosensitive channels conferred
134 protection from hypoosmotic shock/downshock. Because FTL_1753 and FTL_0945 were found
135 in all four strains of *F. tularensis* subspecies *holarctica*, marker-less, in-frame deletions of these
136 two genes were generated in the Live Vaccine Strain (Fig. S4). A hypoosmotic shock of 300mM
137 had little impact on the viability of WT or ΔFTL_0945 strains, but killed >90% of ΔFTL_1753
138 cells, indicating this gene is required for bacterial survival following an osmotic downshock (Fig.
139 1A). To determine the magnitude of osmotic shock required to kill ΔFTL_1753 in media, we
140 measured survival after graded downshocks of 100, 200 or 300mM. The downshock of 300mM
141 killed the vast majority ofΔFTL_1753 cells as before; 200mM downshock caused a moderate but
142 significant loss of viability, and a 100mM downshock did not result in any loss of viability (Fig.
143 1B). These data for ΔFTL_1753 are similar to prior observations for *E. coli* lacking two
144 mechanosensitive channel genes, *mscS* and *mscL* (36). *Trans*-complementation with an
145 expression plasmid for FTL_1753 completely restored the ability of ΔFTL_1753 to survive all
146 three graded downshocks (Fig. 1B). Finally, we tested whether the observed phenotype was
147 relevant to surviving the transition from a mammalian host to fresh water, by equilibrating
148 bacteria in PBS then diluting them into natural lake water or double distilled water. While
149 ΔFTL_1753 was unable to survive in lake water and distilled water, wild-type, ΔFTL_0945, and
150 *trans*-complemented strain of ΔFTL_1753 were able to survive in both conditions (Fig. 1C).
151 These data demonstrate that FTL_1753, but not FTL_0945, encodes a functional
152 mechanosensitive channel that is required by *F. tuluarensis* subsp. *holarctica* to survive
153 hypoosmotic shock, including that encountered when the bacterium transitions from a
154 mammalian host to fresh water.
155

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157

158 ***ΔFTL_1753 bacteria are not more sensitive to detergents, H_2O_2 or antibiotics.***

159 To establish whether deletion of FTL_1753 or FTL_0945 resulted in more generalized
160 vulnerability to stressors, we tested these strains for sensitivity to three detergents and H_2O_2. The
161 three detergents included an anionic detergent (SDS), a cationic detergent (CTAB), and a
162 nonionic detergent (Triton-X100). In contrast to the large differences observed in survival of
163 downshock, all strains displayed a similar sensitivity profile to these stressors (Fig. 2). As
164 mechanosensitive channels have been associated with antibiotic sensitivity in some contexts (41–
165 43), we determined the minimum inhibitory concentrations (MICs) of 9 antibiotics for these
166 strains. The knockout strains displayed identical sensitivity as the wild-type, with the exception
167 of ΔFTL_1753 appearing slightly more resistant to kanamycin (Table S1). These data support a
168 specific role for MscS in survival of hypoosmotic shock, rather than a more general role in
169 resistance of physical or chemical stressors.

170

171 ***MscS does not contribute to virulence in the host niche, but is required to survive the***
172 ***transition from that niche to fresh water.***

173 We next sought to determine the roles of FTL_1753 and FTL_0945 in the context of the
174 mammalian host niche. As *F. tularensis* is an intracellular pathogen that predominantly targets
175 macrophages, we tested the ability of these strains to proliferate in macrophages using
176 gentamicin protection assays. Similar intra-macrophage growth was observed for all strains,
177 suggesting that these genes do not contribute to *F. tularensis* replication within host cells (Fig.
178 3A). For the most part, mammalian tissues are maintained at similar osmolarities. However,

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179 some large osmotic gradients are found in mammals, most notably in the Kidneys and Liver. To
180 determine whether FTL_1753 or FTL_0945 contribute to the pathogenesis of *F. tularensis* in the
181 mammalian host, we tested these mutants in a mouse model of pneumonic tularemia. Mice
182 infected with all strains displayed similar survival profiles, suggesting that these genes do not
183 contribute to virulence within a host (Fig. 3B). Finally, we tested whether FTL_1753 is required
184 for surviving the transition from a mammalian cell niche to fresh water, using modified
185 gentamicin protection assay. After eliminating extracellular bacteria, infected macrophages were
186 lysed with either detergent in PBS, or with ddH_2O. Similar numbers of viable bacteria were
187 recovered using the PBS-detergent lysis buffer. In contrast, FTL_1753 was required for bacteria
188 to survive macrophage lysis with ddH_2O (Fig. 3C). Collectively, these data suggest that MscS is
189 not required for virulence within the mammalian host niche, but is required to survive the
190 transition from this niche to fresh water.
191

192 ***ΔFTL_1753 cells are smaller in average size compared to the wild-type.***

193 As there are reports of mechanosensitive channels affecting cellular morphology (41), and we
194 observed that ΔFTL_1753 often formed smaller colonies than the wild-type, we examined wild-
195 type and ΔFTL_1753 cells using scanning electron microscopy. ΔFTL_1753 cells were of
196 smaller average size than the wild-type; 2D outline traces revealed significantly smaller areas
197 and perimeters compared to the wild-type (Fig. 4A-F). Although smaller in average size,
198 ΔFTL_1753 cells had a similar overall morphology to the wild-type, and there was no difference
199 in the aspect ratio of fitted elliptical axes (Fig. 4G). These data show that the absence of
200 FTL_1753 leads to smaller average bacterial cell size, but does not lead to other gross
201 morphological changes compared to wild-type *F. tularensis* LVS.

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202

203 ***Comparative bioinformatics reveal heterogeneity in the mechanosensitive channels encoded***
204 ***by F. tularensis subspecies***.

205 We chose to focus our study on the *holarctica* subspecies of *F. tularensis*, as this is the
206 subspecies for which an aquatic cycle has been described, and which is responsible for outbreaks
207 of tularemia from contaminated water. Nonetheless, as there is great interest in the highly
208 virulent subsp. *tularensis* and also subsp. *novicida*, we compared the loci predicted to code for
209 mechanosensitive channels in these subspecies to the loci present in subsp. *holarctica*. Of the
210 four loci, the one corresponding to FTL_1753 was most highly conserved between the three
211 subspecies, with only 1-2 amino acid substitutions between them (Fig. 5A and S5). Interestingly,
212 the equivalent of FTL_1753 in the prototypical subsp. *tularensis* (Schu S4) genome is currently
213 annotated as a pseudogene. However, our data and analyses suggest this is erroneous and should
214 be corrected as explained in the discussion. The remaining three loci display marked
215 heterogeneity between the *F. tularensis* subspecies. In the set of loci equivalent to FTL_0945,
216 the C-terminal 44% of the protein is lacking in both ssp. *holarctica* and *tularensis* relative to ssp.
217 *novicida* (Fig. 5B and S6). In another set, the N-terminal ~25% is absent in subsp. *holarctica*
218 relative to the others (Fig. 5C and S7). In the final set, a frameshift has resulted in a premature
219 stop codon and pseudogene annotation in subsp. *holarctica* (Fig. 5D and S8). Overall, these data
220 suggest that: 1) the mechanosensitive channel found to protect *F. tularensis* subspecies
221 *holarctica* from hypoosmotic shock in this study is conserved between the *F. tularensis*
222 subspecies; and 2) the other loci predicted to code for mechanosensitive channels exhibit
223 considerable heterogeneity between the subspecies.

224

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225 **Discussion**

226 The contamination of fresh water by *F. tularensis* subsp. *holarctica* is relevant to the bacterium's
227 ecological persistence and transmission to hosts, including humans. *F. tularensis* can only persist
228 in fresh water for a limited time (27, 28), and is seasonally recovered from natural bodies of
229 water, suggesting recurrent cycles of contamination (7, 31). Early soviet researchers reported
230 that a single infected animal could contaminate 500,000L of water (44), and infected animals are
231 indeed implicated in virtually all cases of water contamination with *F. tularensis* (6–8, 10–12,
232 16, 17, 19, 22, 26, 31–33). To survive an osmotic downshock, such as that encountered when
233 going from a mammalian host to fresh water, bacteria rely on mechanosensitive channels which
234 act as 'pressure relief valves'. In this study, we have identified a mechanosensitive channel
235 which protects *F. tularensis* subsp. *holarctica* from hypoosmotic shock (FTL_1753). The
236 survival profile of ΔFTL_1753 upon downshocks of 100-300mM (Fig. 1B) closely resembles
237 that of *E. coli* lacking both *mscL* and *mscS* (36), and is consistent with the notion that this is the
238 single downshock-protective mechanosensitive channel in the subspecies. We demonstrated that
239 the results of this study are applicable to clinical and environmental strains of subsp. *holarctica*
240 (supplementary Fig. 1-3), and that bacteria lacking FTL_1753 are unable to survive the transition
241 from the osmolarity of the mammalian host to fresh water (Fig. 1C and 3C). Notably, FTL_1753
242 has been found to be up-regulated within the intracellular growth niche(45); speculatively this
243 may be to increase chances of survival in the event of a shift to a freshwater niche.

244

245 The presence of *F. tularensis* subsp. *holarctica* in fresh water has led to outbreaks of mostly
246 oropharyngeal tularemia in at least 10 countries, as described earlier. Oropharyngeal tularemia is
247 often initially misdiagnosed, and without appropriate treatment, it can become a chronic

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248 debilitating disease with the risk of serious complications (23). In addition, the aquatic cycle of
249 subsp. *holarctica* can be implicated in many cases of ulceroglandular tularemia; water is thought
250 to facilitate the spread of bacteria between animals and to mosquito vectors. In cases where
251 humans develop ulceroglandular tularemia from mosquito bites, mosquitos may acquire the
252 bacterium from water during their development. In Sweden, where most cases of tularemia are
253 associated with mosquito bites (3, 46), a strong association has been found between infection and
254 recreational areas near water (47). Furthermore, mosquito larvae reared in pond water from
255 endemic areas, or experimentally exposed to *F. tularensis* during development, have been shown
256 to harbor *F. tularensis* as adults (48, 49). In cases where humans develop ulceroglandular
257 tularemia from handling infected animals, *F. tularensis* can often be found in water within the
258 animals' habitat (26, 33, 50); the presence of viable bacteria in the water is thought to contribute
259 to the spread and persistence of infection among animal populations (26, 33). Thus, the
260 mechanosensitive channel identified here is likely to be important to the overall ecological
261 persistence of *F. tularensis* and its transmission to humans and other susceptible hosts.
262
263 Bioinformatically, we identified four loci predicted to contain mechanosensitive channel
264 domains in each *F. tularensis* genome surveyed, including in subsp. *tularensis* and subsp.
265 *novicida* (Fig. 5, S1-S3, S5-S8). Notably, FTL_1753 was the most conserved between the
266 subspecies, with only one amino acid substitution between ssp. *holarctica* and *tularensis* (Fig.
267 5A, S5). At 165 amino acids, FTL_1753 is in the smallest 2% of the mechanosensitive channel
268 superfamily, which contains over 69,000 members. It is also considerably smaller than MscS in
269 the model organisms in *E. coli* and *B. subtilis*, which are approximately 280 amino acids. This
270 has led to a 'partial' annotation in the non-redundant protein record for FTL_1753

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271 (WP_003017262.1), and a pseudogene annotation in the manually curated subsp. *tularensis*
272 SchuS4 genome. Our data demonstrate that despite its small size, this gene does indeed code for
273 an active mechanosensitive channel that protects from downshock. The other loci containing
274 mechanosensitive channel domains exhibited considerable heterogeneity between subspecies.
275 The functions of these remain to be determined, but in subsp. *holarctica* may include roles other
276 than osmotic shock protection. For instance, other members of the superfamily have been shown
277 to play roles in signal transduction (51).
278
279 In conclusion, we have identified a small, 165 amino acid mechanosensitive channel that protects
280 *F. tularensis* subsp. *holarctia* from downshock. This channel is required for the bacterium to
281 survive the transition from mammalian hosts to fresh water, and likely contributes to ecological
282 persistence of *F. tularensis* and transmission between hosts.
283

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284 **Materials and Methods**

285

286 ***Bioinformatic analysis of msc-domain containing genes in F. tularensis***. The amino acid
287 sequences of mechanosensitive channels (MscS & MscL) that protect *E. coli* from downshock
288 (36) were retrieved from UniprotKB, and input into the Conserved Domain Architecture
289 Retrieval Tool (CDART) (37). Results were filtered to the *Francisella* genus using the NCBI
290 taxonomy tree to identify genes containing msc domains in the strains of interest. ORF and gene
291 sequences were retrieved from RefSeq genomes, compared using standard protein BLAST and
292 Needleman-Wunsch global alignment, and visualized using Multiple Align Show
293 (http://www.bioinformatics.org/sms/multi_align.html).

294

295 ***Bacterial strains and growth conditions***. *Francisella tularensis* subsp. *holarctica* Live Vaccine
296 Strain (LVS) was obtained from Albany Medical College and was grown on MH chocolate agar
297 (Mueller-Hinton II agar (BD BBL) supplemented with 1% hemoglobin (remel) and 1%
298 isovitalex (BD BBL)). Liquid cultures of *F. tularensis* were routinely grown in Brain Heart
299 Infusion Broth, Modified (BHI, BD BBL #299070) at pH 6.4-6.8. Cloning procedures were
300 performed using DH5α *E. coli* grown in LB broth, Miller (BD Difco) or LB agar, Miller (EMD
301 chemicals). Liquid cultures were incubated at 37°C in an orbital shaker operating at 200rpm.
302 Agar plates were incubated at 37°C, 5% CO_2 for 48-72 hours.

303

304 ***Primers, plasmids and DNA manipulation.*** All primers used in this study are shown in table 1.
305 The pMP812 *sacB* suicide vector (38) was used to generate targeted, marker-less, in-frame
306 deletions of desired genes. Briefly, 700-900 base pair PCR products flanking the sequence to be

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307 removed were amplified, digested and ligated together, then re-amplified as one unit and cloned
308 into the mcs of pMP812. A complementation plasmid for *mscS* was generated by modifying the
309 pKK214-GFP plasmid (29). First, the GroEL promoter and GFP gene were removed by digestion
310 with *Xba*I and *Eco*RI. A multiple cloning site with four restriction sites was then added by
311 annealed oligo cloning, with phosphorylated MCS_4_1 & _2 oligonucleotides, to generate
312 pKK214-MCS_4. The bacterioferritin promoter and FTL_1753 were amplified, digested and
313 ligated together, then re-amplified as one unit and cloned into pKK214-MCS_4 with EcoRI. The
314 resulting plasmid is referred to as p-1753. All oligonucleotides were synthesized by Integrated
315 DNA Technologies or the Penn State Genomics Core Facility. All enzymes were from New
316 England Biolabs. $Vent_R$ DNA polymerase was used for cloning and Taq DNA polymerase for
317 screening. Genomic DNA was isolated from *F. tularensis* using an E.Z.N.A. Bacterial DNA Kit
318 (Omega Bio-Tek) and plasmid DNA was isolated using an E.Z.N.A Plasmid DNA Mini Kit I
319 (Omega Bio Tek).
320
321 ***Generation of in-frame deletions via allelic exchange and trans-complementation***. Marker-
322 less, in-frame deletions of FTL_1753 (new locus tag FTL_RS08965) and FTL_0945 (new locus
323 tag FTL_RS04830) were generated using the pMP812 *sacB* suicide vectors as previously
324 described (38). PCR was used to screen for secondary recombinants with deletions, as outlined in
325 Fig. S4. *Trans*-complementation of FTL_1753 was achieved by introducing the p-1753 plasmid
326 into a confirmed ΔFTL_1753 strain. Electrocompetent *F. tularensis* cells were prepared as
327 previously described (39), except that electroporations were done using 0.1cm gap cuvettes
328 (VWR) and Bio-Rad micropulser using Ec1 settings (1.8kV, 10μF and 600Ω).
329

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330 ***Hypoosmotic shock assays***. For downshock experiments conducted in media, single colonies of
331 each strain were picked from MH-chocolate agar plates and grown in BHI supplemented with
332 300mM NaCl (EMD chemicals). Mid-log cultures were diluted (in BHI +300mM NaCl) to a
333 concentration of approximately $5x10^5$ CFU/mL, then 20µL of each culture added to 980µL of
334 BHI +300, +200, +100 or +0 mM NaCl, to produce hypoosmotic shocks of 0, 100, 200 and
335 300mM, respectively. The resulting cultures were vortexed and incubated for 1hr at room
336 temperature, before plating on MH chocolate agar with the same concentrations of NaCl added
337 to prevent further osmotic shock. Percent survival was calculated by comparing viable counts
338 from cultures subject to downshock to cultures maintained with 300mM NaCl throughout the
339 experiment. For downshock experiments from PBS to fresh water, a similar method was used,
340 except that no NaCl was added to growth media, and cells were equilibrated in PBS for 30
341 minutes before dilution into fresh water for 10 minutes. To test a source of water more naturally
342 relevant than double-distilled H_2O, water was retrieved from a freshwater lake in Pennsylvania
343 and passed through a 0.2µm filter.
344

345 ***H_2O_2 and detergent sensitivity assays***. Single colonies of each strain were picked from MH-
346 chocolate agar plates and grown in BHI. Early-log phase cultures were diluted to a concentration
347 of approximately $4x10^4$ CFU/mL, and added 1:1 to plain media or media with 2X the final
348 desired concentration of detergent or H_2O_2. All dilutions and challenges were performed in
349 identical media, such that the only variable would be the detergent/ H_2O_2. These cultures were
350 vortexed and incubated for 1 hour at room temperature, before being diluted 1/10 in BHI, and
351 100µL plated to enumerate viable CFUs. Percent survival was calculated relative to the negative
352 control plates for the same strain (which yielded ~200 CFU).

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353 ***Determination of antibiotic minimum inhibitory concentrations***. 2-fold serial dilutions of each
354 antibiotic were prepared and added to 96 well plates. Bacterial cultures were grown overnight,
355 diluted to an OD_{600} of 0.050, and added 1:1 to plate wells containing media/antibiotics, in
356 triplicate. Plates were incubated overnight (~18h), and bacterial growth determined by measuring
357 optical density at 600nm. The MIC was defined as the lowest final concentration of antibiotic for
358 which cultures did not grow beyond an OD_{600} of 0.1.
359
360 ***Intra-macrophage replication and macrophage-to-freshwater downshock assays.*** Raw 264.7
361 macrophages were maintained in complete DMEM (DMEM +10% FBS, 1mM sodium pyruvate,
362 2mM L-glutamine, 10mM HEPES, 1X non-essential amino acids). For intra-macrophage
363 replication assays, Raw 264.7 macrophages were seeded at a concentration of $3x10^5$ cells per
364 well in 24 well tissue culture plates (Greiner Bio One) the evening before the assay.
365 Macrophages were infected with early-log, BHI-grown *F. tularensis* at a multiplicity of infection
366 of 100 bacteria per macrophage. Plates were centrifuged to facilitate synchronized infection (10
367 minutes, 300 *x g*, RT). After a one hour incubation to allow for bacterial uptake, media was
368 aspirated and replaced with media containing 100µg/mL gentamicin (Gibco) to kill extracellular
369 bacteria. Following an additional one hour incubation, cells were washed twice with PBS. For
370 wells designated for later time points, fresh media (without antibiotic) was added. Cells in other
371 wells were lysed by the addition of 100µL/well of 0.1% w/v sodium deoxycholate (sigma) in
372 PBS. After lysis was observed, 900µL of PBS was added to each well, and serial dilutions plated
373 on MH chocolate agar to determine the number of bacteria that had been taken up by
374 macrophages. 20 hours post-infection, cells were similarly lysed and plated to determine the
375 number of bacteria present. Macrophage-to-freshwater downshock assays were done in a similar

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376 fashion but with the following alterations: $5x10^4$ cells were seeded per well to minimize the
377 contribution of intracellular salt carryover during lysis, and cells were lysed at the 2 hour
378 timepoint only using 200µL/well of either ddH_2O or PBS +0.1% w/v sodium deoxycholate.
379
380 ***Mouse infections***. C57BL/6J mice were maintained in specific pathogen-free conditions at the
381 Pennsylvania State University animal care facilities. Isoflurane-anesthetized mice (n=8 per
382 group) were infected intranasally with 10,000 CFU of bacteria in 50µL of PBS. Serial dilutions
383 of the inoculum were plated on MH chocolate agar to confirm the correct dose was administered.
384 Body weight was measured daily, and mice that lost 20% or more of their starting body weight
385 were euthanized and counted as having succumbed to infection, per Institutional Animal Care
386 and Use Committee (IACUC) guidelines. Experimental groups were age and sex matched.
387
388 ***Electron Microscopy***. 5mL bacterial cultures were grown to an OD_{600} of approximately 0.5 and
389 rinsed twice by centrifuging at 1500 x *g* for 4 minutes then resuspending in pre-warmed media.
390 Cells were fixed by resuspending in 2% glutaraldehyde (Electron Microscopy Sciences) in PBS
391 for 30 minutes at room temperature, then overnight at 4°C. The cell suspension was applied onto
392 Poly-L-Lysine coated coverslips for 10 min at 4°C and proceeded for critical point drying in a
393 chamber of Leica EM CPD300 (Leica Microsystems, Buffalo Grove, IL) critical point dryer
394 through an automatic program. The coverslips were then mounted onto a double-sided carbon
395 tape on a single pin aluminum SEM stab (Ted Pella, Redding, CA) and sputter coated with
396 iridium on a rotation stage of Leica ACE600 (Leica Microsystems, Buffalo Grove, IL). The
397 bacterial samples were examined and imaged in a Zeiss Sigma FE-SEM (Carl Zeiss Microscopy,

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398 Thornwood, NY) at 2 kV. The images were analyzed to determine cell 2D area, perimeter length,
399 and the aspect ratio of a fitted ellipse using ImageJ v1.50i(40).
400
401 ***Statistics***. Statistical differences were determined as indicated in figure legends using GraphPad
402 Prism 5.0.
403
404 ***Ethics statement***. All animal experiments were carried out by following recommendations and
405 approval from the Pennsylvania State University Animal Care and Use Committee (protocol
406 46070) with great care taken to minimize suffering of animals.
407
408
409
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557 **Figure Legends**

558 **Figure 1:** FTL_1753 is required for *F. tularensis* subsp. *holarctica* to survive hypoosmotic
559 shock. **A-B)** Survival data from downshock experiments performed in BHI broth. Bacteria were
560 grown in BHI + 300mM NaCl , then diluted in BHI +300, +200, +100 or +0 mM NaCl,
561 producing hypoosmotic shocks of 0, 100, 200 and 300mM, respectively. **C)** survival data for
562 PBS-equilibrated bacteria diluted into water retrieved from a freshwater lake or double-distilled
563 H_2O. Statistical differences were determined by one-way ANOVA with Dunnett's post test,
564 *p<0.05, **p<0.01, ***p<0.001. Each experiment was performed a minimum of three times.
565

566 **Figure 2:** Sensitivity profiles of wild-type, ΔFTL_1753 and ΔFTL_0945 to detergents and
567 H_2O_2. Early log phase bacteria were exposed to the indicated concentrations of detergents/H_2O_2
568 in media for one hour before dilution and plating to enumerate viable CFUs. Representative
569 results shown from a minimum of four repeats. No statistically significant differences were
570 found by one-way ANOVA with Dunnett's post-test.
571

572 **Figure 3:** FTL_1753 does not contribute to virulence in the host niche, but is required to survive
573 the transition from that niche to fresh water. **A)** Viable CFUs of indicated *F. tularensis* strains
574 recovered from Raw 264.7 macrophages in a gentamicin protection assay at 2 and 20 hours post-
575 infection, pooled data from three repeats. **B)** Survival curves of C57BL/6J mice (n=8 per group)
576 infected with 10^4 CFU of the indicated strains of *F. tularensis* LVS. **C)** Viable CFUs of indicated
577 *F. tularensis* strains recovered from infected RAW 264.7 macrophages based on lysis buffer
578 used. PBS-deox: 0.1% (w/v) sodium deoxycholate in PBS. One-way ANOVA with Tukey's
579 post-test, *p<0.05, ** p<0.01.

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580

581 **Figure 4:** ΔFTL_1753 cells are smaller in average size compared to the wild-type. **A-B)** SEM
582 images of wild-type (A) and ΔFTL_1753 (B) cells at 3,000X. Scale bars: 5μm. **C-D)** SEM
583 images of wild-type (C) and ΔFTL_1753 (D) cells at 10,000X. Scale bars: 1μm. **E-G)** Cells
584 (n>180 per group) were traced in Image J and subjected to the indicated analyses. E) 2-
585 dimensional area of cell traces. F) the perimeter length of cell traces. G) the aspect ratio of
586 ellipses fitted to cell traces. Two-tailed, unpaired t-test, ****p<0.0001. Negative staining
587 experiments with uranyl acetate and phosphotungstic acid followed by TEM yielded similar
588 results.

589

590 **Figure 5**: Summary of comparative alignments of loci predicted to encode mechanosensitive
591 channels in *F. tularernsis* subspecies. White lines represent amino acid substitutions relative to
592 subsp. *holarctica* except where no subsp. *holarctica* sequence is aligned. Gray shading signifies
593 a current pseudogene annotation in RefSeq. For alignment detail, see supplementary Fig. 5-8.

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603 **Table 1**: Primers and plasmids used in this study.

Name	Sequence (5'-3')	Description
1753_UP_F	TATTAAGCGGCCGCAATGTGTGAAATAACTTTTATACCACTTTTAGCAGAT	For upstream flank in FTL_1753 deletion construct
1753_UP_R	GAAGCGGAATTCACTATTGCCTAATTGATCGAACATCGC	For upstream flank in FTL_1753 deletion construct
1753_DOWN_F	TCCCCGGAATTCTAATGCAAAAACGTTACTTTCTTCAGATGAAATCC	For downstream flank in FTL_1753 deletion construct
1753_DOWN_R	AATCTTGTCGACTGCTCACGGAACTCCTCATTTGAGTC	For downstream flank in FTL_1753 deletion construct
0945_UP_F	TATTAAGTCGACCTTTCAGGTAAGACATCAGCAG	For upstream flank in FTL_0945 deletion construct
0945_UP_R	TATTAAGGATCCAACGTTGATATTATGTTGAAAAGCATTTCATT	For upstream flank in FTL_0945 deletion construct
0945_DOWN_F	TATTAAGGATCCTAGGATGTATGAAACTATTGAGCATGG	For downstream flank in FTL_0945 deletion construct
0945_DOWN_R	ATTATGCGGCCGCTGGCATTATTGCTATTGATATATTCACTG	For downstream flank in FTL_0945 deletion construct
0945_SCREEN_F	AGCAAATACCAAACAACGATAACTGCT	For screening for FTL_0945 deletion
0945_SCREEN_R	TCTTTTAATTAATTGCTCAAGCTTTTGGGCA	For screening for FTL_0945 deletion
pBFR_F	GCTCGTCTAGAGATCCATACCCATGATGGTTACTATTG	Bacterioferritin Promoter for plasmid complementation
pBFR_R	GCCGCGGGATCCTATTGTTACCTCCATTATTTAAAACTCTAATCA	Bacterioferritin Promoter for plasmid complementation
1753_COMP_F	TCATCAGGATCCATGTTCGATCAATTAGGCAATAGTGG	For FTL_1753 plasmid complementation
1753_COMP_R	GCTCCGAATTCACCTGATAATAAAAAATAACGCTCATCACT	For FTL_1753 plasmid complementation
MCS_4_1	CTAGAGAATTCGGATCCG	Phosphorylated oligo, for generation of pKK214-MCS4 via annealed oligo cloning

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MCS_4_2	AATTCGGATCCGAATTCT	Phosphorylated oligo, for generation of pKK214-MCS4 via annealed oligo cloning
pMP812		sacB allelic exchange vector, from (38)
pKK214		Expression/complementation plasmid, from (29)

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Primer name	Sequence (5'-3')	Description
1753_UP_F	TATTAAGCGGCCGCAATGTGTGAAATAACTTTTATACCACTTTTAGCAGAT	For upstream flank in FTL_1753 deletion construct
1753_UP_R	GAAGCGGAATTCACTATTGCCTAATTGATCGAACATCGC	For upstream flank in FTL_1753 deletion construct
1753_DOWN_F	TCCCCGGAATTCTAATGCAAAAACGTTACTTTCTTCAGATGAAATCC	For downstream flank in FTL_1753 deletion construct
1753_DOWN_R	AATCTTGTCGACTGCTCACGGAACTCCTCATTTGAGTC	For downstream flank in FTL_1753 deletion construct
0945_UP_F	TATTAAGTCGACCTTTCAGGTAAGACATCAGCAG	For upstream flank in FTL_0945 deletion construct
0945_UP_R	TATTAAGGATCCAACGTTGATATTATGTTGAAAAGCATTTCATT	For upstream flank in FTL_0945 deletion construct
0945_DOWN_F	TATTAAGGATCCTAGGATGTATGAAACTATTGAGCATGG	For downstream flank in FTL_0945 deletion construct
0945_DOWN_R	ATTATGCGGCCGCTGGCATTATTGCTATTGATATATTCACTG	For downstream flank in FTL_0945 deletion construct
0945_SCREEN_F	AGCAAATACCAAACAACGATAACTGCT	For screening for FTL_0945 deletion
0945_SCREEN_R	TCTTTTAATTAATTGCTCAAGCTTTTGGGCA	For screening for FTL_0945 deletion
pBFR_F	GCTCGTCTAGAGATCCATACCCATGATGGTTACTATTG	Bacterioferritin Promoter for plasmid complementation
pBFR_R	GCCGCGGGATCCTATTGTTACCTCCATTATTTAAAACTCTAATCA	Bacterioferritin Promoter for plasmid complementation
1753_COMP_F	TCATCAGGATCCATGTTCGATCAATTAGGCAATAGTGG	For FTL_1753 plasmid complementation
1753_COMP_R	GCTCCGAATTCACCTGATAATAAAAAATAACGCTCATCACT	For FTL_1753 plasmid complementation
MCS_4_1	CTAGAGAATTCGGATCCG	Phosphorylated oligo, for generation of pKK214-MCS4 via annealed oligo cloning
MCS_4_2	AATTCGGATCCGAATTCT	Phosphorylated oligo, for generation of pKK214-MCS4 via annealed oligo cloning
pMP812		sacB allelic exchange vector, from (38)
pKK214		Expression/complementation plasmid, from (29)

Table 1: Primers and plasmids used in this study.

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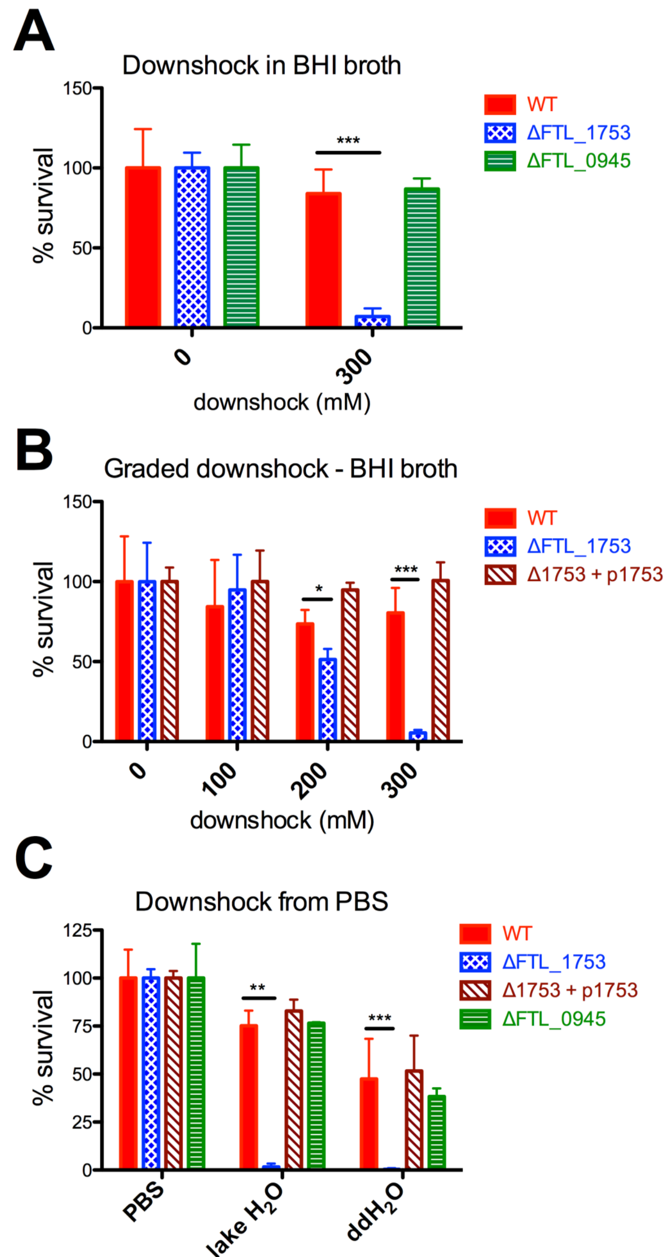

Figure 1: FTL_1753 is required for *F. tularensis* subsp. *holarctica* to survive hypoosmotic shock. **A-B)** Survival data from downshock experiments performed in BHI broth. Bacteria were grown in BHI + 300mM NaCl , then diluted in BHI +300, +200, +100 or +0 mM NaCl, producing hypoosmotic shocks of 0, 100, 200 and 300mM, respectively. **C)** survival data for PBS-equilibrated bacteria diluted into water retrieved from a freshwater lake or double-distilled H_2O. Statistical differences were determined by one-way ANOVA with Dunnett's post test, *p<0.05, **p<0.01, ***p<0.001. Each experiment was performed a minimum of three times.

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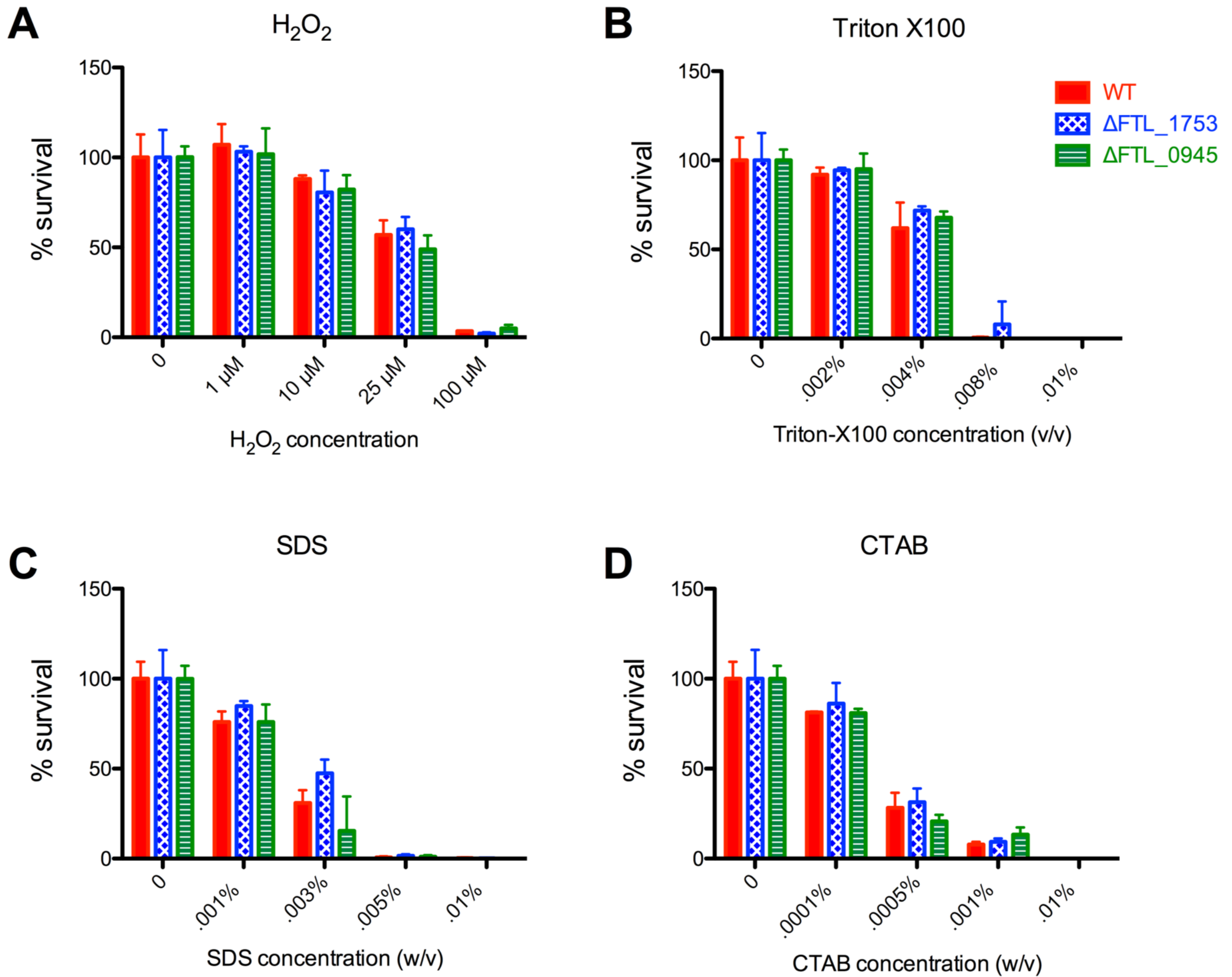

Figure 2: Sensitivity profiles of wild-type, ΔFTL_1753 and ΔFTL_0945 to detergents and H_2O_2. Early log phase bacteria were exposed to the indicated concentrations of detergents/H_2O_2 in media for one hour before dilution and plating to enumerate viable CFUs. Representative results shown from a minimum of four repeats. No statistically significant differences were found by one-way ANOVA with Dunnett's post-test.

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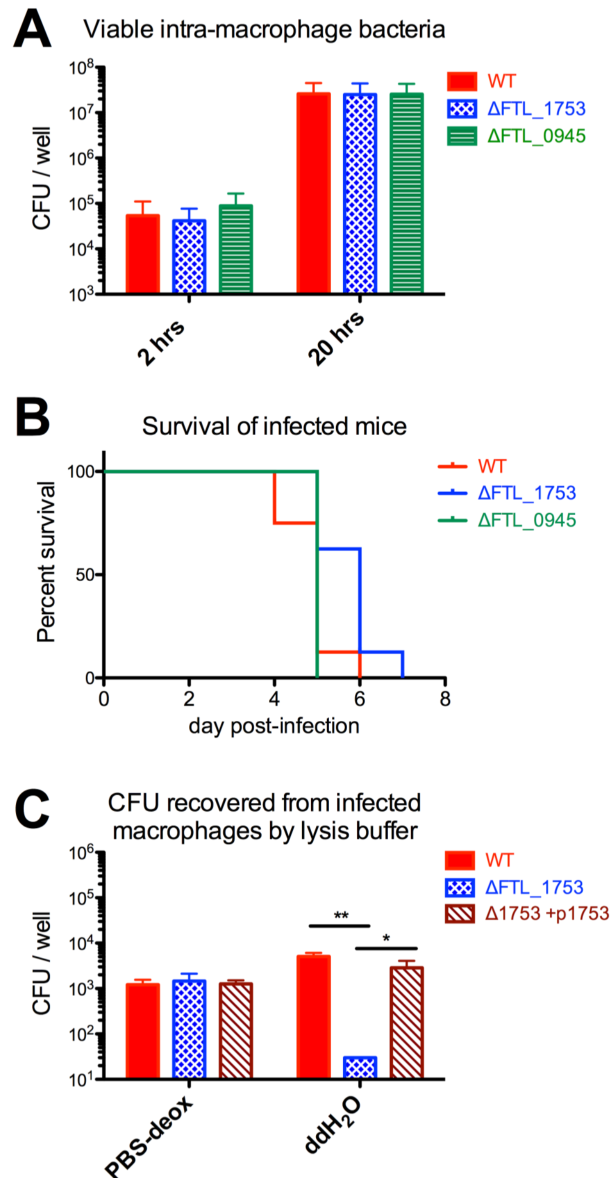

Figure 3: FTL_1753 does not contribute to virulence in the host niche, but is required to survive the transition from that niche to fresh water. **A)** Viable CFUs of indicated *F. tularensis* strains recovered from Raw 264.7 macrophages in a gentamicin protection assay at 2 and 20 hours post-infection, pooled data from three repeats. **B)** Survival curves of C57BL/6J mice (n=8 per group) infected with 10^4 CFU of the indicated strains of *F. tularensis* LVS. **C)** Viable CFUs of indicated *F. tularensis* strains recovered from infected RAW 264.7 macrophages based on lysis buffer used. PBS-deox: 0.1% (w/v) sodium deoxycholate in PBS. One-way ANOVA with Tukey's post-test, *p<0.05, ** p<0.01.

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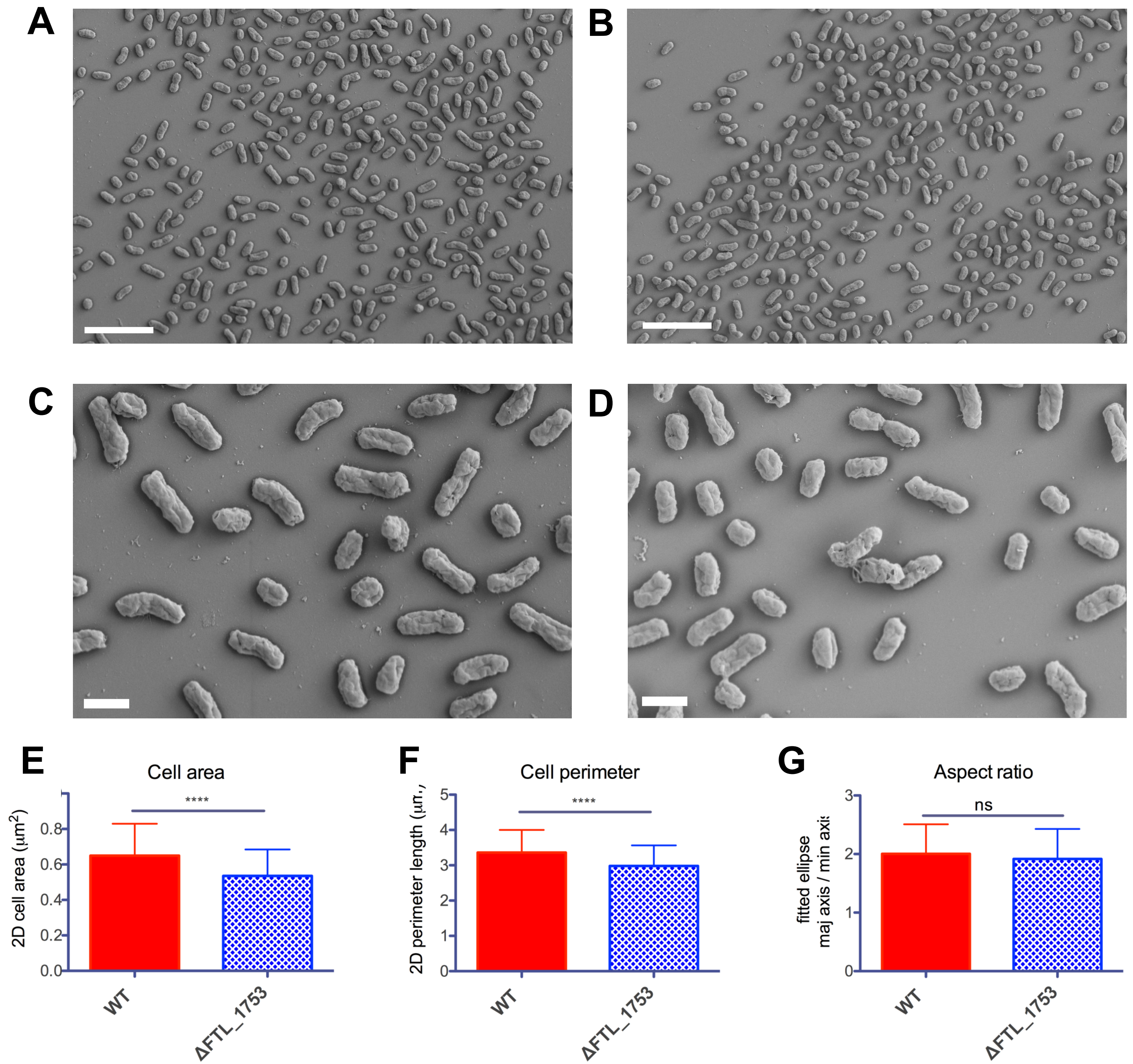

Figure 4: ΔFTL_1753 cells are smaller in average size compared to the wild-type. **A-B)** SEM images of wild-type (A) and ΔFTL_1753 (B) cells at 3,000X. Scale bars: 5µm. **C-D)** SEM images of wild-type (C) and ΔFTL_1753 (D) cells at 10,000X. Scale bars: 1µm. **E-G)** Cells (n>180 per group) were traced in Image J and subjected to the indicated analyses. E) 2-dimensional area of cell traces. F) the perimeter length of cell traces. G) the aspect ratio of ellipses fitted to cell traces. Two-tailed, unpaired t-test, ****$p<0.0001$. Negative staining experiments with uranyl acetate and phosphotungstic acid followed by TEM yielded similar results.

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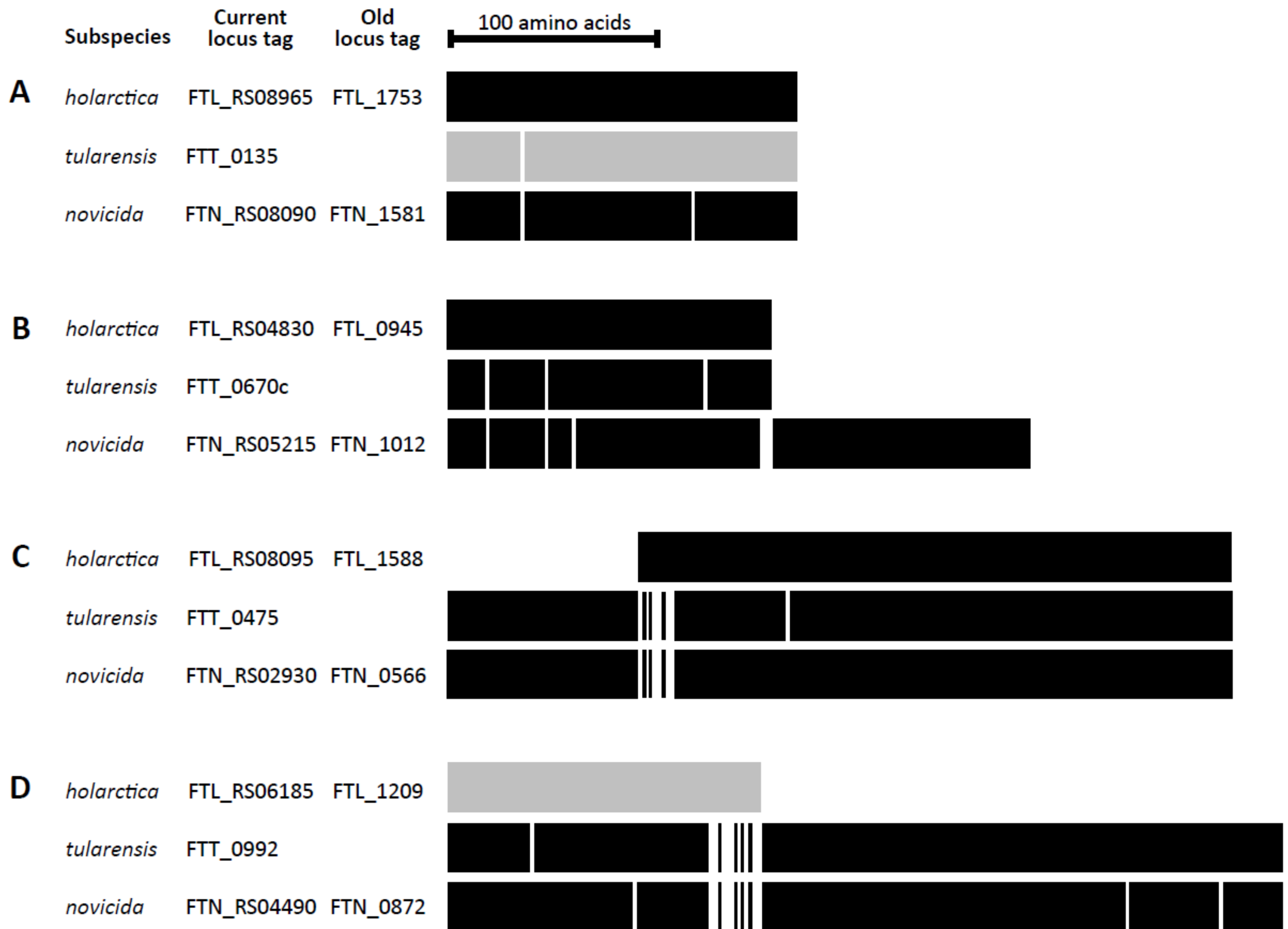

Figure 5: Summary of comparative alignments of loci predicted to encode mechanosensitive channels in *F. tularernsis* subspecies. White lines represent amino acid substitutions relative to subsp. *holarctica* except where no subsp. *holarctica* sequence is aligned. Gray shading signifies a current pseudogene annotation in RefSeq. For alignment detail, see supplementary figures 5-8.