A *Francisella tularensis* L,D-carboxypeptidase plays important roles in cell morphology, envelope integrity, and virulence.

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Running Head: *F. tularensis* L,D-carboxypeptidase

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Summary

Francisella tularensis is a Gram-negative, intracellular bacterium that causes the zoonotic disease tularemia. Intracellular pathogens, including *F. tularensis*, have evolved mechanisms to survive in the harsh environment of macrophages and neutrophils, where they are exposed to cell membrane-damaging molecules. The bacterial cell wall, primarily composed of peptidoglycan (PG), maintains cell morphology, structure, and membrane integrity. Intracellular Gram-negative bacteria protect themselves from macrophage and neutrophil killing by recycling and repairing damaged PG – a process that involves over 50 different PG synthesis and recycling enzymes. Here, we identified a PG recycling enzyme, L,D-carboxypeptidase A (LdcA), of *F. tularensis* that is responsible for converting PG tetrapeptide stems to tripeptide stems. Unlike *E. coli* LdcA and most other orthologs, *F. tularensis* LdcA does not localize to the cytoplasm and also exhibits L,D-endopeptidase activity, converting PG pentapeptide stems to tripeptide stems. Loss of *F. tularensis* LdcA led to altered cell morphology and membrane integrity, as well as attenuation in a mouse pulmonary infection model and in primary and immortalized macrophages. Finally, an *F. tularensis ldcA* mutant protected mice against virulent Type A *F. tularensis* SchuS4 pulmonary challenge.

Keywords: tularemia, peptidoglycan, L,D-carboxypeptidase, virulence, *Francisella tularensis*

Introduction

The Gram-negative bacterial cell wall plays an important role in maintaining cell shape, protecting against external insults, and preventing cell lysis amid fluctuations in internal turgor pressure (Dhar, 2018). The cell wall is composed of peptidoglycan (PG), a network of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) glycan chains that are crosslinked through peptide stems, and lies just outside of the cytoplasmic membrane of most bacteria (Johnson, 2013; Mengin-Lecreulx & Lemaitre, 2005). In *Escherichia coli*, PG has been shown to be covalently attached to the outer membrane (OM) by Braun's lipoprotein and noncovalently attached by Pal and other lipoproteins (Bouveret, 1999; Braun, 1975; Braun, 1969; Leduc, 1992). A loss of membrane integrity can occur if interactions between PG and attached lipoproteins are disturbed, thus maintenance of correct PG architecture is extremely important (Braun & Hantke, 2019; Braun & Rehn, 1969).

Synthesis of Gram-negative PG precursors, studied mainly in *E. coli*, occurs in the bacterial cytoplasm and requires a series of enzymes to build a pentapeptide PG monomer before transporting this molecule into the periplasm. Periplasmic PG cross-linking most often occurs between the fourth residue (D-Ala) of newly-formed pentapeptide stems to the third residue (*meso*-A_2pm) of existing tripeptide stems (4-3 cross links), resulting in the release of the pentapeptide terminal D-Ala and forming tetrapeptide stems (Glauner, 1988; Pazos & Peters, 2019). PG is not a static structure, rather, PG degradation/remodeling is necessary to incorporate new PG and expand the cell wall during bacterial growth and replication, to insert flagella or secretion systems, and to septate during bacterial division (Scheurwater, 2008). Up to 60% of PG is recycled per generation, helping to repair damaged PG and providing energy during periods of stress or starvation (Dhar, 2018; Holtje, 1998; Park, 2008). Using *E. coli* as a model, more than 50 different PG synthesis and hydrolysis/recycling enzymes have been identified, most of which appear to be

71 cytoplasmic. While deletion studies in *E. coli* have indicated that some PG synthesis enzymes are
72 essential, few PG hydrolases have been found to be essential and, in fact, substantial redundancy
73 in hydrolase activity appears to exist. Indeed, alterations in cell morphology, membrane integrity,
74 and ability to replicate/divide sometimes are observed only after multiple PG metabolism genes
75 have been deleted (Dhar, 2018; van Heijenoort, 2011; Vollmer & Bertsche, 2008). Given the
76 increasing threat of antimicrobial-resistant organisms and link between PG homeostasis and
77 bacterial virulence, more studies are needed to understand how a diverse range of Gram-negative
78 bacteria synthesize and recycle PG (Juan, 2018). As proof that PG studies can reveal new
79 information about bacterial pathogenesis and new treatment options, an *Acinetobacter baumannii*
80 penicillin-binding protein (PBP) mutant was reported to be more sensitive to complement-
81 mediated killing than wild-type bacteria (Russo, 2009) and a *Helicobacter pylori* PG hydrolase
82 (AmiA) mutant was unable to colonize mouse stomachs (Chaput, 2016).

83 PG recycling, mainly characterized in *E. coli,* begins with periplasmic lytic
84 transglycosylase (LT) cleavage of the β-1,4-glycosidic linkage between MurNAc and GlcNAc,
85 forming a GlcNAc-1,6-anhydro-MurNAc product, which allows for insertion of new muropeptides
86 and recycling of the GlcNAc-1,6-anhydro-MurNAc peptide (Scheurwater, 2008). Low molecular
87 mass penicillin binding proteins (LMM PBPs) can function as endopeptidases, cleaving the cross-
88 links between adjacent tetrapeptide stems, and/or as D,D-carboxypeptidases, removing the
89 terminal D-Ala of pentapeptides during transpeptidation (cross-linking) reactions, forming the
90 tetrapeptide (Dhar, 2018). Inner membrane permeases, such as AmpG, then transfer GlcNAc-1,6-
91 anhydro-MurNAc disaccharides, with or without attached peptides, to the cytoplasm where they
92 can be disassembled. Cytoplasmic NagZ and AmpD further degrade disaccharides by cleaving
93 the bond between GlcNAc and 1,6-anhydro-MurNAc, and separating the peptide chain from 1,6-
94 anhydro-MurNAc, respectively. Additional cytoplasmic hydrolases, such as L,D-

95 carboxypeptidases (Ldc), act on free peptide chains to cleave the terminal D-Ala from
96 tetrapeptides, resulting in tripeptides (Dhar, 2018). PG is unusual in that it is both highly dynamic
97 (*e.g.,* allowing for bacterial division and molecular transport across the periplasm), yet tightly
98 regulated to prevent membrane collapse and bacterial death. As such, PG recycling enzymes have
99 been speculated to be important virulence determinants in Gram-negative bacteria (Juan, 2018).
100 Indeed, *E.coli ldc* mutants lyse in stationary phase (Templin, 1999) and are more susceptible to β-
101 lactam antibiotics (Ursinus, 1992), *Helicobacter* and *Campylobacter ldc* mutants have altered cell
102 morphology and defects in motility and biofilm formation (Frirdich, 2012; 2014; Sycuro, 2013),
103 and *Neisseria gonorrhoeae ldc* mutants are unable to stimulate NOD1-dependent responses in the
104 host (Lenz, 2017). However, very little is known about the importance of PG recycling enzymes
105 in the pathogenesis of intracellular pathogens such as *Francisella tularensis*.

106 *F. tularensis*, the causative agent of tularemia, is a Gram-negative, intracellular,
107 coccobacillus that can infect and cause lethal disease in many species, including humans (Dennis,
108 2001; Keim, 2007). There are three subspecies of *F. tularensis*, subsp. *tularensis* (Type A), subsp.
109 *holarctica* (Type B), and subsp. *mediasiatica*, although only subsp. *tularensis* and subsp.
110 *holarctica* are virulent for humans (Kingry, 2014). *F. tularensis* poses a severe threat to public
111 health and has been classified as an NIH Category A Priority Pathogen and a CDC Tier 1 Select
112 Agent due to its low infectious dose (<10 CFU), ease of aerosolization, and high morbidity and
113 mortality rates (up to 60%) (Ellis, 2002; Sjostedt, 2007). Like other intracellular pathogens, *F.*
114 *tularensis* has evolved different mechanisms to infect, survive, and replicate within host cells,
115 including macrophages and neutrophils (Ray, 2009). However, this lifestyle exposes the bacteria
116 to reactive oxygen species (ROS), reactive nitrogen species (RNS), antimicrobial peptides, and
117 other cell membrane- and cell wall-damaging molecules (Jones, 2012). Our group previously
118 demonstrated that the *F. tularensis* disulfide bond formation protein A (DsbA) ortholog repairs

119 damaged outer membrane proteins and known virulence factors. We additionally showed that *F.*
120 *tularensis* DsbA, unlike periplasmic DsbA in *E. coli* and most other Gram-negative bacteria, is
121 outer membrane-bound and is a multifunctional protein with both oxidoreductase and isomerase
122 activities. Finally, using a molecular trapping approach, we identified over 50 *F. tularensis* DsbA
123 substrates, many of which we speculate are involved in virulence (Ren, 2014).

124 Here, we determined the function of one of those *F. tularensis* DsbA substrates – a
125 previously unstudied hypothetical protein containing a putative LdcA domain – and assessed its
126 role in bacterial virulence. Deletion of *F. tularensis* LdcA resulted in bacteria with altered cell
127 morphology, increased sensitivity to β-lactam antibiotics, yet increased resistance to several
128 stressors (*e.g.,* H_2O_2, NaCl, low pH). Next, we demonstrated that *F. tularensis* LdcA exhibits L,D-
129 carboxypeptidase and L,D-endopeptidase activities on pentapeptide and tetrapeptide residues of
130 PG. Finally, we established that *F. tularensis* LdcA is required for virulence, as mutants were
131 unable to replicate in macrophages or cause disease in mice.

132

133 **Results**

134 *FTL1678 contains a putative L,D-carboxypeptidase domain*

135 Previous studies by our group and others have shown that *F. tularensis* DsbA mutants are
136 attenuated in mice (Qin, 2009; Ren, 2014). However, additional work by our group, demonstrating
137 that DsbA possesses both oxidoreductase and isomerase activities to repair damaged envelope and
138 cell membrane proteins, indicated that other envelope proteins likely are responsible for *F.*
139 *tularensis* virulence (Ren, 2014). To identify new *F. tularensis* virulence factors, we used a
140 molecular trapping approach and identified over 50 *F. tularensis* DsbA substrates (Ren, 2014).
141 One of those DsbA substrates, FTL1678, is annotated in the *F. tularensis* genome as a conserved
142 membrane hypothetical protein. Here, a conserved domain search revealed that a large portion of

143 FTL1678 contains a putative Ldc domain, part of the peptidase_S66 superfamily (Figure S1). Ldc
144 proteins have been studied in a number of Gram-negative bacteria, including *E. coli* (Metz, 1986a;
145 b; Templin, 1999; Ursinus, 1992), *Pseudomonas aeruginosa* (Korza, 2005), *N. gonorrhoeae* (Lenz,
146 2017), and *Campylobacter jejuni* (Frirdich, 2014). To further explore this conserved domain,
147 amino acid sequences of FTL1678 (*F. tularensis* subsp. *holarctica* [Type B] LVS) and FTT0101
148 (homolog of FTL1678 in *F. tularensis* subsp. *tularensis* [Type A] SchuS4) were aligned with LdcA
149 orthologs from *E. coli*, *P. aeruginosa*, *N. gonorrhoeae*, and *C. jejuni* (named Pgp2). Despite low
150 percentages of amino acid identities among the LdcA orthologs (6.3% to 30.3%; Figure 1), there
151 was a higher degree of amino acid similarity among LdcA orthologs (13.0% [*E. coli* and *C. jejuni*]
152 to 44.7% [*E. coli* and *N. gonorrhoeae*]; Figure 1). Notably, the LdcA Ser-Glu-His catalytic triad,
153 previously shown to be required for *P. aeruginosa* LdcA activity (Korza, 2005), was absent from
154 *C. jejuni* Pgp2 but was present in all LdcA homologs, including FTL1678 and FTT0101 (Figure
155 1).

156 *E. coli* and *P. aeruginosa* Ldcs have been localized to the bacterial cytoplasm (Korza, 2005;
157 Templin, 1999). However, *C. jejuni* Pgp2 is unusual in that it contains a signal peptide and has
158 been speculated to be periplasmic (Frirdich, 2014). In addition, *N. gonorrhoeae* LdcA was found
159 to be periplasmic and outer membrane-associated (Lenz, 2017). As noted above, we previously
160 demonstrated that FTL1678 is a DsbA substrate (Ren, 2014), indicating that FTL1678 is located
161 in the *F. tularensis* envelope (*i.e.,* in the inner membrane [IM], periplasm, or outer membrane
162 [OM]). Bioinformatic analyses of FTL1678 indicated that it is a periplasmic protein due to the
163 presence of a signal peptide but absence of OM or lipoprotein signatures (Table S1). To
164 experimentally confirm FTL1678 localization, we generated an *F. tularensis* strain with 6×
165 histidine-tagged FTL1678, then performed spheroplasting, osmotic lysis, and sucrose density
166 gradient centrifugation to separate IM and OM fractions and probe for protein subcellular

167 localization. Immunoblotting of whole-cell lysates (WCL), OM fractions, and IM fractions
168 demonstrated that the OM control protein, FopA (Huntley, 2007), only was present in WCL and
169 OM fractions (but not IM fractions; Figure 2) and the IM control protein, SecY (Huntley, 2007),
170 only was present in WCL and IM fractions (but not OM fractions; Figure 2). By comparison,
171 FTL1678 only was detected in WCL and OM fractions, demonstrating OM-association (Figure 2).
172 As noted above, because *N. nonorrhoeae* LdcA was found to fractionate to both the OM and
173 soluble fractions (indicating periplasmic localization) (Lenz, 2017), we next examined the
174 localization of *F. tularensis* periplasmic proteins in our fractions to better understand FTL1678
175 localization. TolB is a well-known periplasmic protein in Gram-negative bacteria and binds to PG
176 with the peptidoglycan associated lipoprotein, Pal (Clavel, 1998; Walburger, 2002). We
177 previously demonstrated that the *F. tularensis* Pal homolog is OM-localized (Huntley, 2007),
178 similar to its OM-localization in other Gram-negative bacteria. Here, the *F. tularensis* TolB
179 homolog was detected in OM fractions, but not IM fractions (Figure S2), demonstrating that some
180 *F. tularensis* periplasmic proteins fractionate with OMs. Additional fractionation and periplasmic
181 protein localization (Jones et al, 2016) experiments were performed but specific localization of
182 FTL1678 to the periplasm could not be confirmed (data not shown). Regardless, data that
183 FTL1678 is a DsbA substrate (*i.e.,* FTL1678 is an envelope protein)(Ren, 2014), FTL1678
184 contains a signal peptidase I cleavage site (Table S1), FTL1678 does not contain membrane protein
185 signatures (Table S1), and FTL1678 is OM-associated (Figure 2), provides strong evidence that
186 FTL1678 is not a cytoplasmic protein, unlike *E. coli* LdcA. Instead, our data indicate that, similar
187 to *N. nonorrhoeae* LdcA, FTL1678 may be a periplasmic protein.
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191 *FTL1678 exhibits L,D-carboxypeptidase and L,D-endopeptidase activities*

192 To confirm the predicted Ldc activity of FTL1678 and FTT0101, recombinant FTL1678
193 and FTT0101 were expressed and affinity purified from *E. coli*. As a control, lysates from *E. coli*
194 containing the empty vector (pPROEX HTb) also were affinity-purified . Recombinant FTL1678,
195 lysate from the vector control, or buffer alone were incubated with various PG precursors and PG
196 intermediates (Table 1) to determine substrate specificity and specific activity. The vector control
197 and buffer alone did not demonstrate activity against any of the PG substrates (data not shown).
198 When FTL1678 was incubated with various PG substrates, the highest specific activity was
199 detected against the tetrapeptide substrates GlcNAc-anhydroMurNAc-L-Ala-γ-D-Glu-*meso*-
200 A_2pm-D-Ala (tracheal cytotoxin; TCT; 21.5 nmol/min/mg of protein) and GlcNAc-MurNAc-L-
201 Ala-γ-D-Glu-*meso*-A_2pm-D-Ala (reducing PG monomer; 15.6 nmol/min/mg of protein),
202 confirming that FTL1678 exhibits L,D-carboxypeptidase activity (Table 1). Interestingly,
203 FTL1678 activity against free tetrapeptide, L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala, was approx. 6-fold
204 lower (3.4 nmol/min/mg of protein) than TCT and 5-fold lower than the reducing PG monomer
205 (Table 1), indicating that GlcNAc and MurNAc may be important for tetrapeptide recognition or
206 FTL1678 binding. Importantly, FTL1678 exhibited specific activity against pentapeptide
207 substrates MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (9.8 nmol/min/mg of protein;
208 Table 1) and UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (5.9 nmol/min/mg of
209 protein; Table 1), indicating that FTL1678 also functions as an L,D-endopeptidase (cleavage of
210 the pentapeptide between *meso*-A_2pm and D-Ala) by FTL1678.

211 To further investigate the endopeptidase activity of FTL1678, FTL1678 was incubated
212 with various TCT monomer and dimer substrates, containing different peptide lengths and cross-
213 link locations (Table S2). Although FTL1678 exhibited the highest specific activity against TCT
214 monomers containing tetrapeptide stems with either an Alanine or a Glycine in the fourth position

215 (20 nmol/min/mg and 18 nmol/min/mg of protein, respectively), FTL1678 also was able to cleave
216 all four variations of the TCT dimer (two cross-linked TCT monomers), demonstrating
217 endopeptidase activity (Table S2). Of the four different TCT dimers tested, FTL1678 was most
218 active on TCT dimers connected by a 4-3 cross linkage (6.6 nmol/min/mg of protein), followed by
219 an approx. 24-fold reduction in activity on 3-3 cross links connecting two tripeptides (0.28
220 nmol/min/mg of protein), a tripeptide and tetrapeptide with a Glycine at the fourth position (0.21
221 nmol/min/mg of protein), and a tripeptide and tetrapeptide with an Alanine at the fourth position
222 (0.16 nmol/min/mg of protein) (Table S2). These data suggest that, while FTL1678 exhibits
223 endopeptidase activity on 4-3 and 3-3 cross-links, cleavage of 3-3 cross links is likely not its main
224 physiological function.

225 Finally, FTL1678 had negligible activity against L-lysine-containing substrates (0.7 to 1.3
226 nmol/min/mg of protein; Table 1), where L-lysine replaced *meso*-A_2pm at the third amino acid
227 position, indicating the importance of *meso*-A_2pm. Assays were repeated with recombinant
228 FTT0101 (SchuS4 homolog) and, due to 99.4% amino acid identify with FTL1678, FTT0101
229 demonstrated similar tetrapeptide cleavage activity (*i.e.,* LdcA activity) as FTL1678 (Table 1).
230 FTT0101 also was not active on a peptidoglycan polymer and had either no or negligible activity
231 on PG monomers that were amidated at the *meso*-A_2pm or D-Glu residues (Table 1). Additionally,
232 FTT0101 was not inhibited by 5 mM EDTA and did not require the presence of cations (Mg^{2+}) for
233 tetrapeptide cleavage (data not shown).

234

235 *FTL1678 controls bacterial morphology*

236 LdcA has been shown to be important for maintenance of bacterial morphology and
237 structural integrity (Frirdich, 2014; Sycuro, 2013). In addition, mutations/deletions or
238 combinations of mutations/deletions in PG-modifying proteins can result in abnormal bacterial

239 morphology, emphasizing the importance of PG modification and recycling (Guinane, 2006;
240 Heidrich, 2001; Juan, 2018; Nelson, 2000; Priyadarshini, 2007; Sycuro, 2010). To assess if
241 FTL1678 plays a similar role in *F. tularensis*, we generated an isogenic deletion of *FTL1678*,
242 referred to hereafter as Δ*FTL1678*, in *F. tularensis* LVS. When examined by transmission electron
243 microscopy (TEM), wild-type (WT) bacterial width ranged from 350 to 800 nm (Figures 3A and
244 3D), whereas Δ*FTL1678* bacteria were more uniform in cell width, averaging approx. 350 nm
245 (Figures 3B and 3D). WT bacteria were observed to be coccobacilli with loosely-associated OM
246 (Figure 3A), while Δ*FTL1678* bacteria were found to be more coccoid in appearance, the OM was
247 tightly-associated, and three prominent structures were present around the periphery of each
248 bacterium, likely the OM, PG, and IM (Figure 3B). Additionally, Δ*FTL1678* bacteria appeared
249 more electron dense and had a significantly-thicker OM than WT bacteria (Figures 3A, 3B, and
250 3C).

251 Previous studies have shown that deletion of genes for PG-modifying proteins (*e.g.,* murein
252 hydrolases) can result in abnormal growth characteristics, including lysis during stationary phase
253 (Templin, 1999) and an inability to separate daughter cells at the septa during cell division,
254 resulting in abnormal bacterial chains (Chaput, 2016; Denome, 1999; Heidrich, 2001; 2002; Juan,
255 2018; Priyadarshini, 2007; 2006). Although *N*-acetylmuramyl-L-alanine amidases have been
256 shown to be predominantly involved in the cleavage of bacterial septa, deletion of lytic
257 transglycosylases and some endopeptidases, in combination with amidase deletions, also have
258 resulted in abnormal bacterial chains (Heidrich, 2001; 2002). To examine any potential replication
259 defects of Δ*FTL1678*, we compared both OD_{600} values (Figure S3A) and CFUs over time (Figure
260 S3B) of WT and Δ*FTL1678* in supplemented Mueller Hinton Broth (sMHB; standard growth
261 medium for *F. tularensis*; (Huntley, 2007)), finding that Δ*FTL1678* did not have any inherent
262 growth defects. When examining both WT and Δ*FTL1678* by TEM for any septation defects or

263 abnormal bacterial chains, approximately 10% of Δ*FTL1678* bacteria grew in chains of three to
264 four bacteria (Figure S4A and S4B), whereas no WT bacteria exhibited this septation defect (data
265 not shown). Taken together, our findings that Δ*FTL1678* is 1.5- to 2-times smaller than WT
266 (Figure 3D), Δ*FTL1678* is more coccoid in shape (Fig 3B), Δ*FTL1678* has a pronounced three-
267 layered envelope, including a prominent electron dense layer between the IM and OM (likely PG;
268 Figure 3B), and Δ*FTL1678* has a partial septum defect (Figure S4), further support the role of
269 FTL1678 as a PG-modifying enzyme that is important for bacterial elongation and division.
270
271 *Deletion of FTL1678 affects antibiotic, detergent, and stressor sensitivity*
272 Given the above noted morphological differences in the Δ*FTL1678* envelope (*e.g.,* thicker
273 OM; tightly-associated OM; three prominent envelope structures) using TEM, we assessed
274 envelope (*i.e.,* IM, PG, and OM) integrity by growing both WT and Δ*FTL1678* bacteria in the
275 presence of various antibiotics, detergents, and dyes, and measuring zones of inhibition after 48 h
276 of growth (Table 2). Δ*FTL1678* was found to be more susceptible than WT to ampicillin,
277 vancomycin, lysozyme, and SDS (Table 2), indicating potential changes to PG (ampicillin
278 sensitivity), OM integrity (vancomycin and lysozyme sensitivity), or efflux pumps (SDS
279 sensitivity). Conversely, Δ*FTL1678* was found to be more resistant than WT to gentamicin,
280 tetracycline, chloramphenicol, ciprofloxacin, ethidium bromide, and Triton X-100 (Table 2).
281 Given that the majority of these latter reagents must enter the cytoplasm to exert their inhibitory
282 effects (*i.e.,* gentamicin, tetracycline, and chloramphenicol inhibit protein synthesis; ciprofloxacin
283 and ethidium bromide interfere with DNA replication), these results suggest that Δ*FTL1678*
284 bacteria exclude these inhibitory molecules from entering the cytoplasm.
285 To better understand potential differences in the Δ*FTL1678* envelope, WT and Δ*FTL1678*
286 were grown in either sMHB at 37°C or in sMHB with various stress conditions. In sMHB at 37°C,

287 Δ*FTL1678* did not exhibit a growth defect but, instead, appeared to grow to a higher optical density
288 (OD_{600}) than WT (Figure 4A). However, as noted above, despite higher OD_{600} measurements for
289 Δ*FTL1678* at several time points, bacterial numbers were not significantly different between WT
290 and Δ*FTL1678* (Figure S3). Although speculative, the disassociation between Δ*FTL1678* optical
291 densities and bacterial numbers may be due to the observed TEM morphological differences of
292 Δ*FTL1678* (Figure 3 and Figure S4). Compared with growth in sMHB at 37°C, no substantial
293 differences in the growth rates of WT and Δ*FTL1678* were observed at either 40°C or in the
294 presence of 60 μM $CuCl_2$ (Figure 4B and 4C). However, Δ*FTL1678* grew considerably better than
295 WT in the presence of 5 mM H_2O_2, 5% NaCl, and pH 5.5 (Figure 4D, 4E, and 4F, respectively),
296 providing further evidence of modifications to the Δ*FTL1678* envelope. Although many
297 mechanisms can account for increased resistance to H_2O_2, high NaCl, and low pH, the combined
298 results from these antibiotic, detergent, and stressor sensitivity/resistance assays suggest that
299 Δ*FTL1678* bacteria may have decreased OM integrity, altered PG, altered activity of efflux pumps,
300 and a less permeable IM. Indeed, decreased permeability of the IM and altered activity of IM
301 efflux pumps may help explain the more electron-dense staining of Δ*FTL1678*, compared to WT
302 (Figure 3).
303
304 *Δ*FTL1678 is fully-attenuated in a mouse pulmonary infection model*

305 To examine if FTL1678 plays a role in *F. tularensis* virulence, mice were intranasally
306 infected with 10^4 CFU of either WT or Δ*FTL1678* and monitored daily for signs of disease.
307 Whereas all WT-infected mice died by day 9 post-infection (median time-to-death day 6),
308 Δ*FTL1678* was completely attenuated (100% survival through day 21 post-infection),
309 demonstrating that FTL1678 is required for *F. tularensis* virulence (Figure 5A). To confirm that
310 the observed attenuation was solely due to the deletion of *FTL1678*, and not to polar effects, we

311 complemented Δ*FTL1678* with a 6× His-tagged FTL1678 *in trans*, which fully-restored virulence
312 to WT levels (all mice died by day 7; median time-to-death day 6; Figure 5A).

313 To more carefully assess Δ*FTL1678* attenuation *in vivo*, we intranasally-infected mice with
314 10^4 CFU of either WT LVS or Δ*FTL1678*, and enumerated bacterial CFUs from lungs, livers,
315 spleens, and blood on days 2 and 5 post-infection to examine bacterial replication and
316 dissemination to these organs/tissues over time. On day 2 post-infection, WT LVS replicated to
317 $>10^7$ CFU/mg lung and had disseminated to livers, spleens (approx. 10^3 CFU/mg), and blood (10^3
318 CFU/ml; Figure 5B). In contrast, Δ*FTL1678* had an initial (day 2) colonization defect in the lungs
319 (4-logs less than WT) and was unable to disseminate to livers, spleens, or blood (Figure 5B). By
320 day 5 post-infection, the attenuation of Δ*FTL1678* was even more apparent, with WT LVS
321 replicating to extremely high numbers (approx. 10^8 CFU/mg) in lungs, livers, and spleens,
322 compared with Δ*FTL1678,* which replicated approx. 1-log in lungs (between day 2 and 5), but was
323 4-log attenuated in lungs and was not detectable in livers or spleens (Figure 5B). Although
324 Δ*FTL1678* was detected in the blood on day 5, it was 2-logs less than WT LVS (Figure 5B).

325 The Type A *F. tularensis* strain SchuS4 originally was isolated from a human tularemia
326 patient and requires BSL3 containment. Given its relevance to human disease, we next generated
327 an isogenic deletion mutant of the *FTL1678* homolog, *FTT0101*, in SchuS4. When mice were
328 intranasally-infected with either WT SchuS4 or Δ*FTT0101,* all mice died by day 7 post-infection,
329 indicating that *FTT0101* is not required for SchuS4 virulence (Figure S5). Whereas Δ*FTT0101*-
330 infected mice exhibited a slightly delayed time-to-death (median time-to-death day 6; Figure S5),
331 compared with WT SchuS4-infected mice (median time-to-death day 5; Figure S5), this may be
332 due to differences in the infectious dose administered to mice in this experiment (80 CFU SchuS4;
333 12 CFU Δ*FTT0101*). However, it also remains possible that the extreme virulence of SchuS4
334 (intranasal LD_{50} <10 CFU in our hands) complicates assessments of mutant attenuation *in vivo*.

335 To further assess Δ*FTT0101*, the susceptibilities of Δ*FTT0101* and WT SchuS4 to various
336 antibiotics, detergents, and dyes were compared, with no significant differences observed (Table
337 S3). At this time, we cannot fully explain why the *F. tularensis* Type B LdcA mutant Δ*FTL1678*
338 is fully-attenuated in mice (Figure 5) and displays altered sensitivity/resistance to antibiotics,
339 detergents, and dyes (Table 2), while the *F. tularensis* Type A LdcA mutant Δ*FTT0101* retains
340 virulence in mice (Figure S5) and does not demonstrate altered sensitivity/resistance to antibiotics,
341 detergents, and dyes (compared to WT SchuS4; Table S3). However, this finding is not
342 unexpected given genomic studies indicating that, despite >97% nucleotide identity between Type
343 A and Type B *F. tularensis*, there are over 100 genomic rearrangements between Type A and Type
344 B and each subspecies encodes over 100 unique genes that likely influence known differences in
345 Type A and Type B virulence (Petrosino et al, 2006).
346
347 *Putative FTL1678 catalytic triad and Ldc activity are required for F. tularensis virulence*
348 As noted above and highlighted in Figure 1, *P. aeruginosa* LdcA contains a Ser-Glu-His
349 catalytic triad which is essential for function and is characteristic of Ldc in the Peptidase_S66
350 family (Korza, 2005). The Ser-Glu-His catalytic triad also has been confirmed in Ldc from *E. coli*
351 (Meyer, 2018), *Novosphingobium aromaticivorans* (Das, 2013), and *N. gonorrhoeae* (Lenz, 2017).
352 Given the relatively conserved spacing of Ser134-Glu239-His308 residues in FTL1678 (Figure 1),
353 we tested if these residues were required for *F. tularensis* virulence (similar to Figure 5A virulence
354 assessments for Δ*FTL1678* and the FTL1678 complemented strain). Site-directed mutagenesis
355 was performed to independently generate FTL1678 complementation constructs containing either
356 S134A, E239A, or H308A mutations. Next, Δ*FTL1678* was complemented *in-trans* with each of
357 these FTL1678 catalytic triad point mutants, and mice were intranasally infected with either WT,
358 Δ*FTL1678*, Δ*FTL1678 trans*-complemented with *FTL1678*, or Δ*FTL1678 trans*-complemented

359 with one of the FTL1678 catalytic triad point mutants (referred to hereafter as S134A, E239A, and
360 H308A). Confirming our previous findings, Δ*FTL1678* was completely attenuated (100% survival
361 through day 21), while complementation of Δ*FTL1678* with either FTL1678 (all mice dead by day
362 8), S134A (all mice dead by day 7), E239A (all mice dead by day 8), or H308A (all mice dead by
363 day 8), fully-restored virulence to WT LVS levels (all mice dead by day 10; Figure S6). These
364 results indicated that although a putative Ser-Glu-His catalytic triad is present in FTL1678,
365 mutation of individual residues does not affect *F. tularensis* virulence *in vivo*.

366 Given the complexity of *in vivo* animal infections and the possibility that single mutations
367 in the putative Ser134-Glu239-His308 catalytic triad may not be sufficient to abolish enzyme
368 function or alter bacterial virulence, we next assessed if mutations of two or three residues in the
369 putative catalytic triad affected *in vitro* enzyme activity. Recombinant FTL1678 mutant proteins
370 were generated and purified, each containing either two amino acid mutations (S134A/E239A,
371 S134A/H308A, and E239A/H308A) or three amino acid mutations (S134A/E239A/H308A).
372 Similar to what is described above, enzymatic assays were performed, using the TCT monomer as
373 a substrate. While no specific activity to TCT monomer was detected for the empty vector control
374 or buffer alone, WT FTL1678 was active against the TCT monomer (11.0 nmols/min/mg). By
375 comparison, no activity was detected for any of the double or triple mutant proteins (Table S4),
376 suggesting that two or more of the catalytic triad residues are required for FTL1678 Ldc activity.

377 The Ldc ortholog alignment (Figure 1) highlighted that, of the six Ldc orthologs examined
378 here, only *C. jejuni* Pgp2 lacked the Ser-Glu-His catalytic triad. However, *C. jejuni* Pgp2 has been
379 shown to exhibit LdcA activity (Frirdich, 2014), indicating that a Ser-Glu-His catalytic triad is not
380 required for LdcA function. To test if an LdcA ortholog, without the Ser-Glu-His catalytic triad,
381 could restore virulence in the Δ*FTL1678* mutant, we complemented Δ*FTL1678* with a 6× His-
382 tagged Pgp2 from *C. jejuni* and infected mice with either WT LVS, Δ*FTL1678*, Δ*FTL1678 trans*-

383 complemented with FTL1678, or Δ*FTL1678 trans*-complemented with *C. jejuni* Pgp2. While
384 Δ*FTL1678* was fully attenuated (100% survival through day 21), the Pgp2 *trans*-complement was
385 fully-virulent (median time-to-death 6 days; all mice dead by day 7), nearly identical to WT LVS
386 (median time-to-death 7 days; all mice dead by day 7) and the FTL1678 *trans*-complement
387 (median time-to-death 6 days; all mice dead by day7; Figure 5C). This *in vivo* data provides further
388 evidence that FTL1678 is an Ldc and Ldc activity is required for *F. tularensis* virulence.

389 To provide additional evidence that FTL1678 exhibits Ldc activity and that *C. jejuni* Pgp2
390 functionally complements Δ*FTL1678*, the FTL1678 *trans*-complement and Pgp2 *trans*-
391 complement were examined by TEM, revealing that both complemented strains had similar
392 morphology as WT LVS (Figure S7A and S7B; Figure 3A). Additionally, OM thickness and cell
393 width were measured for WT, Δ*FTL1678*, and both complemented strains, demonstrating that both
394 complemented strains had OM thicknesses and cell widths similar to WT, and both complemented
395 strains were significantly different from Δ*FTL1678* (Figure S7C and S7D). Finally, when grown
396 in the presence of various stressors (*e.g.,* 5 mM H_2O_2, 5% NaCl, pH 5.5, antibiotics, SDS,
397 lysozyme, or ethidium bromide), both complemented strains exhibited similar phenotypes as WT
398 LVS (Figure S8A-D and Table S5). Taken together, these studies indicated that at least two
399 residues of the putative Ser134-Glu239-His308 catalytic triad are required for FTL1678 LdcA
400 enzymatic activity. These studies also demonstrated that an LdcA ortholog can functionally
401 complement Δ*FTL1678,* further supporting that FTL1678 is an LdcA ortholog.

402

403 *FTL1678 is required for F. tularensis replication in macrophages*

404 *F. tularensis* is an intracellular pathogen and macrophages appear to be one of the major
405 targets for *F. tularensis* infection and replication (De Pascalis, 2018; Hall, 2008; Steiner, 2017).
406 To investigate potential replication defects of Δ*FTL1678* in macrophages, J774A.1 macrophages

407 or murine bone marrow-derived macrophages (mBMDM) were infected with either WT LVS or
408 Δ*FTL1678* (MOI 100:1) and bacterial numbers were enumerated at 0 h (entry), 6 h, and 24 h post-
409 infection. At entry (0 h), approx. 2.5-logs more Δ*FTL1678* were present in both macrophage lines,
410 compared with WT LVS (Figure 6A). This likely was due to the above noted gentamicin resistance
411 of Δ*FTL1678* (Table 2). Attempts to normalize entry numbers for both WT LVS and Δ*FTL1678*,
412 using different antibiotics or combinations of antibiotics, were not successful. Despite higher
413 numbers of Δ*FTL1678* in both macrophages at entry (0 h) and 6 h, Δ*FTL1678* was unable to
414 replicate in either macrophage, and decreased approx. 1-log from 6 h to 24 h (Figure 6A). By
415 comparison, WT LVS numbers increased 1.5- to 2-logs from 6 h to 24 h (Figure 6A). To normalize
416 WT LVS and Δ*FTL1678* bacterial numbers and replication rates in both macrophages, fold change
417 in bacterial numbers was calculated from 6 h to 24 h: WT was found to increase 2.5- and 1.5-logs
418 in J774A.1 and mBMDMs, respectively, whereas Δ*FTL1678* was found to decrease 0.5- to 1-log,
419 respectively (Figure 6B). Taken together, these *in vitro* results (Figures 6A and 6B) confirm the
420 observed *in vivo* attenuation of Δ*FTL1678* (Figures 5A and 5B).
421
422 *ΔFTL1678 protects mice against Type A F. tularensis infection*
423 No FDA-approved vaccine currently is available to prevent tularemia. In addition, *F.*
424 *tularensis* is designated as an NIH Category A priority pathogen and CDC Tier 1 Select Agent,
425 highlighting the extreme virulence of this bacterium and the need for a safe and effective vaccine
426 to prevent tularemia. Given our above findings that 10^5 CFU of Δ*FTL1678* did not cause disease
427 or death in mice (Figures 5A and 5C), we next examined whether high doses (10^7 or 10^9 CFU) of
428 Δ*FTL1678* were attenuated or if Δ*FTL1678* immunization could protect mice from fully-virulent
429 Type A *F. tularensis* SchuS4 challenge. First, all mice intranasally immunized with either 10^5,
430 10^7, or 10^9 CFU of Δ*FTL1678* survived through day 28 post-infection, with no signs of clinical

431 disease (Figure 7A). Next, on day 29, all mice were boosted with 10^9 CFU of Δ*FTL1678* and no
432 mice demonstrated any signs of disease through day 50 (Figure 7A). Finally, on day 51, mice
433 were intranasally-challenged with 120 CFU (6× the LD_{50}) of SchuS4 and the health status of each
434 immunization group was monitored for 26 days post-challenge. In a dose-dependent manner, the
435 10^9 prime-10^9 boost regimen conferred 80% protection, the 10^7 prime-10^9 boost regimen conferred
436 40% protection, and the 10^5 prime-10^9 boost regimen conferred 20% protection (Figure 7B).
437 These data demonstrate that Δ*FTL1678* is highly attenuated (up to 10^9 CFU) and that Δ*FTL1678*
438 may be able to be used as a live, attenuated vaccine.
439
440 *ΔFTL1678 does not cause tissue damage*
441 The *in vitro* (Figure 6) and *in vivo* (Figures 5A, 5B, 7A) attenuation of Δ*FTL1678,* as well
442 as protection against SchuS4 pulmonary challenge (Figure 7B), indicated that Δ*FTL1678* could be
443 used as a live, attenuated vaccine. While live, attenuated vaccines have been extremely effective
444 at preventing a number of diseases, they can pose safety challenges (Minor, 2015; Roberts, 2018).
445 To assess whether Δ*FTL1678* immunization induced any pathology in immunized mice, lungs,
446 livers, and spleens from uninfected, WT LVS-, or Δ*FTL1678*-infected mice were assessed for
447 pathologic changes on day 5 post-infection/immunization. Day 5 is when mice exhibit severe
448 signs of disease and is one day before the majority of WT-infected mice begin succumbing to
449 disease (Figures 5 and 7). WT LVS-infected lungs demonstrated alveolar wall thickening, large
450 areas of inflammation, and severe neutrophil infiltration (Figure 8A). By comparison, little
451 inflammation was observed in Δ*FTL1678*-infected lungs, although some red blood cell congestion
452 was present, indicating a limited, acute immune response that was quickly resolved (Figure 8A).
453 Whereas WT LVS-infected livers were characterized by diffuse inflammation with focal areas of
454 necrosis, Δ*FTL1678*-infected livers were virtually indistinguishable from uninfected livers, with

455 no observable pathology (Figure 8A). Finally, although the architecture of WT LVS-infected
456 spleens lacked distinct areas of white pulp or red pulp, indicative of a severe infection, Δ*FTL1678*-
457 infected spleens were observed to contain distinct areas of red pulp and white pulp, with some red
458 blood cell congestion – indicating a limited, acute immune response that was quickly resolved
459 (Figure 8A). All tissues were blindly scored using a pathology severity index (scale from 0 to 4,
460 with 4 indicating severe pathology), confirming that Δ*FTL1678*-infected tissues were virtually
461 indistinguishable from uninfected tissues (pathology scores of 1 for lungs, 0 for liver, and 1.5 for
462 spleens) and WT LVS-infected tissues had significantly higher pathology scores (pathology scores
463 >3.5 for all tissues; Figure 8B).
464

465 **Discussion**

466 Bacterial PG is a complex, mesh-like structure, composed of a glycan backbone,
467 crosslinked to varying degrees, by peptide chains (de Pedro, 2015). It is well known that this
468 structure plays an important role in maintaining Gram-negative bacterial cell morphology,
469 membrane integrity, regulating changes in osmotic pressure, and providing a platform for
470 attachment of the OM (den Blaauwen, 2008; Silhavy et al, 2010). Although a majority of PG
471 studies have focused on how the thick layer of PG in Gram-positive bacteria contributes to
472 virulence and antibiotic resistance, more recent studies have highlighted that Gram-negative PG
473 also is intimately linked with pathogenicity (Juan, 2018).

474 PG recycling is an essential function of Gram-negative bacteria during cell growth and
475 division to produce new cell wall components. In fact, Gram-negative bacteria recycle up to 60%
476 of their PG with every generation, suggesting that both PG synthesis and PG recycling are dynamic
477 (Dhar, 2018; Typas, 2011). A number of proteins are involved in these processes and, while they
478 are well-characterized in *E. coli*, very little is known about these pathways in intracellular

479 pathogens such as *Burkholderia pseudomallei*, *Legionella pneumophila*, or *F. tularensis* (Jenkins,
480 2019; Kijek, 2019; Spidlova, 2018; van Heijenoort, 2011).

481 *E. coli* LdcA, a cytoplasmic protein, was the first L,D-carboxypeptidase to be identified
482 and was shown to be important for PG recycling and survival during stationary phase (Templin,
483 1999; Ursinus, 1992). More recently, Ldc orthologs have been identified in *P. aeruginosa* (Korza,
484 2005), *C. jejuni* (Frirdich, 2014), *N. gonorrhoeae* (Lenz et al, 2017), and *N. aromaticivorans* (Das,
485 2013). In this study, we identified an *F. tularensis* Ldc ortholog, FTL1678, which we propose
486 naming LdcA based on its confirmed L,D, carboxypeptidase activity (Table 1) and role in
487 maintaining bacterial morphology. Unlike well-characterized cytoplasmic LdcA orthologs from
488 *E. coli* and *P. aeruginosa*, we demonstrated that *F. tularensis* LdcA was localized to OM fractions
489 and, given co-localization with PG-associated proteins Pal and TolB, is most likely located on the
490 inner leaflet of the OM or in the periplasm (associated with PG). At this time, we can only
491 speculate on the OM-association or periplasmic localization of *F. tularensis* LdcA, but in the
492 context of PG repair and recycling, periplasmic LdcA certainly offers a fitness advantage. In
493 addition, this is not the first report of a periplasmic LdcA, as *C. jejuni* Pgp2 is predicted to be
494 periplasmic and *N. gonorrhoeae* LdcA previously was reported to be periplasmic (Frirdich, 2014;
495 Lenz, 2017).

496 Our results demonstrated, for the first time, that *F. tularensis* LdcA directly acts on the
497 TCT tetrapeptide and the reducing PG monomer. More importantly, we demonstrated that *F.*
498 *tularensis* LdcA directly cleaves PG pentapeptides to tripeptides, without a prior cleavage event
499 by a D,D-carboxypeptidase/penicillin binding protein (PBP), such as DacD, and that FTL1678
500 cleaves TCT dimers with 4-3 and 3-3 cross links, highlighting that *F. tularensis* LdcA is a multi-
501 functional enzyme that performs both L,D-carboxypeptidase and L,D- and D,D-endopeptidase
502 activities (Table 1 and Table S2). . It should be noted that although *N. gonorrhoeae* LdcA has

503 been reported to have L,D-endopeptidase activity, this activity is specific to tetrapeptide cross links
504 (3-3 cross links) (Lenz, 2017). Interestingly, only two previous studies have examined putative *F.*
505 *tularensis* PG modifying enzymes and both studies primarily focused on the role of an *F. tularensis*
506 DacD ortholog in virulence, with no PG activity assays to confirm function (Kijek, 2019; Spidlova,
507 2018). In our PG cleavage analysis, *F. tularensis* LdcA demonstrated the highest specific activity
508 on disaccharide-tetrapeptide PG substrates (GlcNAc-anhydroMurNAc-L-Ala-γ-D-Glu-*meso*-
509 A_2pm-D-Ala [TCT] and GlcNAc-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala [reducing PG
510 monomer]), followed by cleavage of pentapeptide PG substrates (MurNAc-L-Ala-γ-D-Glu-*meso*-
511 A_2pm-D-Ala-D-Ala and UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala). Despite high
512 specific activity of *F. tularensis* LdcA on tetrapeptide attached to the disaccharide, *F. tularensis*
513 LdcA demonstrated approximately 6-times lower specific activity on free tetrapeptide (no sugars)
514 (Table 1). In contrast, *E. coli* LdcA has been shown to have the highest specific activity on free
515 tetrapeptide, monosaccharide-tetrapeptide (MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala), and
516 monosaccharide tetrapeptide linked to a glycan lipid carrier (UDP-MurNAc-tetrapeptide)
517 (Templin, 1999), but is unable to cleave dimeric muropeptides. Additionally, *F. tularensis* LdcA
518 was active against TCT dimers (two crosslinked TCT monomers), cleaving the 4-3 cross linkage
519 between D-Ala and A_2pm and the 3-3 cross linkage between A_2pm and A_2pm, but was not active
520 on PG polymers. This TCT dimer cleavage indicates that, in addition to L,D carboxypeptidase
521 activity, *F. tularensis* LdcA possesses L,D- and D,D-endopeptidase activity on peptide cross-links.
522 Previous studies have shown that Ldc orthologs are important for bacterial morphology
523 and membrane integrity. Deletion of the *ldc* orthologs *csd6* from *H. pylori* (Sycuro, 2013) and
524 *pgp2* from *C. jejuni* (Frirdich, 2014) resulted in loss of helical morphology. Here, we demonstrated
525 that FTL1678 is essential for maintaining both the size (width) and the coccobacillus morphology
526 of *F. tularensis,* as Δ*FTL1678* were significantly-smaller than WT and exhibited a more-rounded,

527 cocci shape than WT (Figure 3). Further evidence for the role of *F. tularensis* LdcA in modifying
528 and recycling PG, which impacts bacterialmorphology, is provided by TEM images of Δ*FTL1678*
529 bacteria that had prominent three-layered structures at their periphery, including a thick middle
530 layer (presumably PG), compared to WT (Figure 3). In Δ*FTL1678* bacteria, it is possible that loss
531 of LdcA activity may have reduced PG recycling or may have affected the breakdown of existing
532 PG (important for cell division), resulting in a buildup of pentapeptides or tetrapeptides that are
533 highly-crosslinked. Our lab and others have repeatedly attempted to isolate and analyze *F.*
534 *tularensis* PG but theses attempts have not been unsuccessful (data not shown; Martin Pavelka, U.
535 Rochester, personal communication). As such, we can only speculate on the true nature of the
536 thick PG and OM layers in Δ*FTL1678* (Figure 3).

537 Because Δ*FTL1678* bacteria were found to have a thicker OM (Figure 3C), a prominent
538 middle layer in their envelope (presumably PG; Figure 3B), and altered cell morphology (Figure
539 3B), we investigated differences in WT and Δ*FTL1678* susceptibility to various antibiotics,
540 detergents, and stressors. Vancomycin and lysozyme, usually not effective against Gram-
541 negatives due to their inability to penetrate the OM, inhibited Δ*FTL1678* growth (Table 2),
542 indicating increased permeability of the Δ*FTL1678* OM. Vancomycin, in particular, may have
543 been effective on Δ*FTL1678* because its mechanism of action includes binding to the two terminal
544 D-Ala-D-Ala residues of PG pentapeptide chains and preventing cross-linking of monomers.
545 Δ*FTL1678* may have increased amounts of pentapeptides present in its PG, providing more targets
546 for vancomycin action. Similarly, ampicillin inhibits bacterial transpeptidases, which blocks
547 cross-linking of peptide side chains of PG strands. Taken together, the enhanced susceptibility of
548 Δ*FTL1678* bacteria to vancomycin and ampicillin supports the role of *F. tularensis* LdcA as a PG-
549 modifying enzyme. Conversely, Δ*FTL1678* was more resistant to antibiotics and molecules that
550 must cross the IM to exert their toxic effects, including gentamicin, tetracycline, chloramphenicol,

551 ciprofloxacin, and ethidium bromide (Table 2), suggesting that Δ*FTL1678* bacteria have a less
552 permeable IM. Mechanisms to explain why Δ*FTL1678* bacteria were more resistant to other
553 stressors, including Triton X-100, H_2O_2, high salt, and low pH, are less clear, but could include
554 increased expression/activity of chaperone proteins, efflux pumps, antioxidant/scavenger proteins,
555 and membrane stabilizing proteins (Knodler et al, 2003; Lund et al, 2014; Mishra & Imlay, 2012).
556 The extreme virulence of Type A *F. tularensis* and its designation as a Tier 1 Select Agent
557 highlight why studies to identify *F. tularensis* virulence factors and the development of new
558 vaccines is important. In this study, we identified the role of a previously unstudied protein,
559 FTL1678, in PG recycling, PG integrity, and bacterial morphology. In addition, we found that
560 FTL1678 was required for *F. tularensis* LVS virulence and demonstrated that Δ*FTL1678*
561 conferred 80% protection against fully-virulent, Type A *F. tularensis* SchuS4 pulmonary
562 challenge. Further studies are needed to determine specific immune responses induced by
563 Δ*FTL1678* immunization, as well as to identify the most effective immunization regimen (*e.g.,*
564 number of immunizations and time between immunizations).
565 Finally, given our findings that PG maintenance and recycling are important for *F.*
566 *tularensis* virulence, and that future studies may reveal additional PG-associated enzymes, we used
567 bioinformatic approaches to predict other proteins involved in *F. tularensis* PG synthesis and
568 recycling (Figure 9). While at least seven PG synthesis and recycling genes/proteins orthologs
569 could not be identified, 22 putative PG synthesis and recycling proteins were identified in *F.*
570 *tularensis* (Figure 9). Of these, only DacD (FTL1060/FTT1029) has been studied in *F. tularensis*.
571 Given our observed attenuation of Δ*FTL1678*, future studies to better understand PG synthesis and
572 recycling pathways may offer more opportunities to better understand the virulence of *F. tularensis*
573 and other intracellular pathogens. Characterization of other proteins involved in PG pathways may

574 provide clues as to why *F. tularensis* LdcA is OM-associated or periplasmic and encodes both
575 L,D-carboxypeptidase and L,D-endopeptidase activities.
576
577
578 **Experimental Procedures**
579 *Bacterial strains and culture conditions*
580 *F. tularensis* Type A strain SchuS4 and *F. tularensis* Type B strain LVS were obtained from BEI
581 Resources and cultured as previously described (Ren, 2014; Wu, 2016). All experiments with
582 SchuS4 were performed under BSL3 containment conditions at the University of Toledo Health
583 Science Campus BSL3 laboratory. Routine *F. tularensis* cultures were grown overnight at 37°C
584 with 5% CO_2 on supplemented Mueller-Hinton agar (sMHA): Mueller-Hinton broth powder
585 (Becton Dickinson) was mixed with 1.6% (wt/vol) Bacto Agar (Becton Dickinson), autoclaved,
586 and further supplemented with 2.5% (vol/vol) bovine calf serum (Hyclone), 2% (vol/vol)
587 IsoVitaleX (Becton Dickinson), 0.1% (wt/vol) glucose, and 0.025% (wt/ vol) iron pyrophosphate.
588 For mouse infections, *F. tularensis* was first grown on sMHA then transferred to Brain Heart
589 Infusion agar (BHI; Becton Dickinson). Chocolate agar for mutant strain generation was prepared
590 by mixing Mueller Hinton broth powder with 1.6% (wt/vol) agar, 1% (wt/vol) tryptone, and 0.5%
591 (wt/vol) sodium chloride, autoclaved, and further supplemented with 1% (wt/vol) hemoglobin and
592 1% (vol/vol) IsoVitaleX. For macrophage infections, *F. tularensis* was first grown on sMHA then
593 transferred to modified chocolate agar: Mueller-Hinton broth powder was mixed with 1.6%
594 (wt/vol) Bacto Agar, 1% hemoglobin (wt/vol), and 1% (vol/vol) IsoVitaleX. All growth curves
595 were performed in sMHB: Mueller-Hinton broth powder was mixed with 182 mg/L calcium
596 chloride dihydrate, and 210 mg L^{-1} magnesium chloride hexahydrate, 0.1% (wt/vol) glucose,
597 0.025% (wt/vol) iron pyrophosphate, and 2% (vol/vol) IsoVitaleX. All bacterial strains and

598 plasmids are listed in Table S6. *E. coli* S17-1 and *E. coli* NEB10-β were grown in Luria Bertani
599 (LB) broth or on LB agar at 37°C, supplemented as needed with antibiotics.
600
601 *Sequence Alignments and Bioinformatic Predictions*
602 Amino acid alignments of *F. tularensis* subsp. *holarctica* FTL_1678, *F. tularensis* subsp.
603 *tularensis* FTT_0101, *E. coli* LdcA (BAA36050.1), *P. aeruginosa* LdcA (Q9HTZ1), *N.*
604 *gonorrhoeae* (YP_208343.1), and *C. jejuni* Pgp2 (WP_002856863) were performed using Clustal
605 Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and MView
606 (https://www.ebi.ac.uk/Tools/msa/mview/). Pairwise sequence alignments were performed and
607 amino acid identities among Ldc homologues were calculated by EMBOSS Needle
608 (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). The Prokaryotic Genome Analysis Tool
609 (PGAT) (http://tools.uwgenomics.org/pgat/), BlastP, and BlastX analyses
610 (http://blast.ncbi.nlm.nih.gov) were used to identify *F. tularensis* homologues. Bacterial protein
611 sub-localization was predicted by PSORTb version 3.0.2 (https://www.psort.org/psortb/). Protein
612 signal sequence prediction was performed by LipoP version 1.0
613 (http://www.cbs.dtu.dk/services/LipoP/) and SignalP version 4.1
614 (http://www.cbs.dtu.dk/services/SignalP-4.1/).
615
616 *Generation of F. tularensis gene deletion mutants*
617 *F. tularensis* isogenic deletion mutants were generated by homologous recombination as
618 previously described (Wu, 2015). Briefly, 500 bp regions upstream and downstream from the gene
619 of interest (*FTL1678* or *FTT0101*) were PCR-amplified from *F. tularensis* genomic DNA using
620 the following primers: FTL1678_A and FTL1678_B; FTL1678_C and FTL1678_D; FTT0101_A
621 and FTT0101_B; FTT0101_C and FTT0101_D (Table S7). A FLP recombination target (FRT)-

622 flanked Pfn-kanamycin resistance cassette, FRT-Pfn-kan-FRT, was PCR amplified from pLG66a
623 (Gallagher, 2008) and splicing overlap extension PCR (SOE PCR) was used to join the upstream
624 (A-B) and downstream (C-D) regions with FRT-Pfn-kan-FRT, which replaced the gene of interest.
625 The resulting insert and a suicide plasmid, pTP163 (Robertson, 2013), were digested with ApaI
626 (New England Biolabs), and ligated using T4 DNA ligase (New England Biolabs). Gene deletion
627 constructs were transformed into NEB10-β *E. coli* (New England Biolabs), sequence-verified,
628 transformed into *E. coli* S17-1, and conjugation was performed with *F. tularensis* LVS on sMHA
629 plates. Conjugants were recovered on chocolate agar supplemented with 200 mg L^{-1} hygromycin
630 and 100 mg L^{-1} polymyxin B. Individual mutants were selected by sequential plating on sMHA
631 supplemented with 10 mg L^{-1} kanamycin (sMHA-kan10), sMHA-kan10 with 8% (wt/vol) sucrose,
632 and final replica plating onto sMHA containing either 200 mg L^{-1} hygromycin (sMHA-hyg200) or
633 sMHA-kan10. Hyg-sensitive and kan-resistant colonies were sequence verified (referred to
634 hereafter as either Δ*FTL1678* or Δ*FTT0101*).
635
636 *FTL1678 complementation in trans*
637 Complementation *in trans* was performed as previously described, with some modifications (Wu,
638 2016). *FTL1678* was PCR-amplified from *F. tularensis* LVS using primers
639 5'FTL1678_NEBuilder and 3'FTL1678_NEBuilder (Table S7), pQE-60 (Qiagen) was double-
640 digested with NcoI and BglII (New England Biolabs), and the NEBuilder HiFi DNA Assembly
641 Cloning kit was used to ligate the *FTL1678* amplicon and digested pQE-60. The construct was
642 transformed into NEB 10-β *E. coli* and transformants were selected on LB agar supplemented with
643 100 mg L^{-1} ampicillin (LB-amp). Plasmids were purified from individual clones using the Qiagen
644 QIAprep Spin Miniprep kit (Qiagen), diagnostic PCR was performed to confirm insert presence
645 and correct size, and DNA sequencing was performed to verify insert integrity. The resulting

646 construct, *FTL1678* with a C-terminal 6x histidine tag, was PCR-amplified using primers
647 5'FTL1678_pFNLTP6 and 3'FTL1678_pFNLTP6 (Table S7), the amplicon and pFNLTP6-gro-
648 GFP (Maier, 2004) were double-digested with XhoI and BamHI (New England Biolabs), and
649 ligated using T4 DNA Ligase. The construct, pFNLTP6-gro-*FTL1678*-6xHis, was transformed
650 into NEB10-β *E. coli*, transformants were selected on LB plates supplemented with 50 mg L^{-1}
651 kanamycin (LB-kan), and DNA sequencing was performed to verify FTL1678-6xHis integrity.
652 Next, the kan resistance gene was removed from Δ*FTL1678* by suspending the strain in 0.5 M
653 sucrose (in 1 mM EDTA, pH 7.5), washing three times, and electroporating the shuttle plasmid
654 pTP405 (Robertson, 2013), which encodes the Flp recombinase to remove FRT-Pfn-kan-FRT from
655 the genome. Bacteria were grown overnight on sMHA-hyg200, hyg-resistant transformants were
656 passaged three times on sMHA, then transformants were replica plated onto sMHA-hyg200 and
657 sMHA-kan10 to confirm sensitivity to both antibiotics (kan-cured Δ*FTL1678*). pFNLTP6-gro-
658 *FTL1678*-6xHis was transformed into kan-cured Δ*FTL1678* by electroporation, transformants
659 were selected on sMHA-kan10, and expression of FTL1678 was confirmed by immunoblot
660 analysis (referred to hereafter as Δ*FTL1678 trans*-complement).
661
662 *C. jejuni Pgp2 complementation in trans*
663 Complementation of *C. jejuni pgp2* (CJJ81176_0915) into Δ*FTL1678* was performed as described
664 above, with several modifications. The *pgp2* gene, with the FTL1678 signal sequence (amino acid
665 residues 1-29) in place of the native Pgp2 signal sequence (amino acid residues 1-18), was
666 synthesized and inserted in pQE-60 by GenScript USA. pQE-60-*pgp2* was transformed into
667 NEB10-β *E. coli* and selection was performed on LB-amp. Pgp2-6xHis was amplified from pQE-
668 60 using primers 5'FTL1678_pFNLTP6 and 3'FTL1678_pFNLTP6 (Table S7), the amplicon was
669 ligated into similarly digested pFNLTP6, pFNLTP6-gro-*pgp2*-6xHis was transformed into

670 NEB10-β *E. coli*, and transformants were selected on LB-kan. Plasmids were purified from kan-
671 resistant transformants, sequence verified, then electroporated into kan-cured Δ*FTL1678*. Pgp2
672 expression was confirmed by immunoblot analysis.
673
674 *Mouse Infections*
675 All animal studies were approved by the University of Toledo Institutional Animal Care and Use
676 Committee (IACUC). Mouse infections were performed as previously described (Huntley, 2008),
677 with some modifications. Briefly, *F. tularensis* strains were grown on sMHA overnight,
678 transferred to BHI agar for an additional 20-24 h, suspended in sterile PBS, and diluted to the
679 desired concentration (20 to 10^9 CFU/20 µl) based on previous OD_{600} measurements and bacterial
680 enumeration studies. Groups of 4-8 female C3H/HeN mice (6-8 weeks old; Charles River
681 Laboratories) were anesthetized with a ketamine-xylazine sedative and intranasally (i.n.) infected
682 with 20 µl of prepared bacterial suspensions. Bacterial innocula were serially-diluted and plated
683 in quadruplet on sMHA to confirm CFUs. For survival studies, mice were monitored daily, for
684 signs of disease, with health status scores (scale of 1-5, with 1 indicating healthy and 5 indicating
685 mouse found dead) being recorded for each mouse. Moribund mice were humanely euthanized to
686 minimize suffering. To quantitate bacterial tissue burdens, groups of 4 mice were euthanized on
687 days 2 and 5 post-infection, blood was collected by cardiac puncture and plated onto sMHA, lungs,
688 livers, and spleens were aseptically harvested, homogenized, 25 µl of PBS/mg of tissue was added
689 to each tissue, serially-diluted, and dilutions were plated onto sMHA. Following 72 h of
690 incubation, the number of colonies per plate were counted and CFU/mg (tissues) or CFU/ml
691 (blood) were calculated based on tissue weight and dilution factor. For immunization and
692 challenge studies, groups of 4-10 mice were i.n. immunized with either 100-300 CFU LVS or 10^4-
693 10^9 CFU ΔFTL1678, boosted 3-4 weeks later with either 10^3 CFU LVS or 10^9 CFU ΔFTL1678,

694 transported to the ABSL3 facility 3-weeks later, and i.n. challenged with 20-120 CFU of *F.*
695 *tularensis* SchuS4. Mice were monitored daily for signs of disease with health status scores being
696 recorded for each mouse.
697
698 *Membrane Integrity Testing*
699 Sensitivity of LVS, Δ*FTL1678*, FTL1678 *trans*-complement, and the Pgp2 *trans*-complement to
700 various antibiotics, detergents, dyes, and cell wall stressors was determined by disk diffusion
701 assays or in liquid cultures, as previously described (Wu, 2016), with some modifications.
702 Bacterial strains were grown on either sMHA or sMHA-kan10 (ΔFTL1678 and complement
703 strains), scraped and resuspended in sterile PBS, adjusted to an OD_{600} of 0.2 (approx. $9x10^7$
704 CFU/ml), diluted 1:1 in PBS, and 100 µl was plated onto sMHA plates using cotton tipped
705 applicators (Puritan). Sterile paper disks (Whatman; 0.8 mm thick, 6.5 mm in diameter) were
706 placed in the center of each plate and antibiotics, detergents, or dyes were added to the disks at the
707 concentrations listed in Table 2. Antibiotics tested were: gentamicin (Gibco), tetracycline (Fisher
708 Scientific), chloramphenicol (Acros Organics), ciprofloxacin (Oxoid), ampicillin (Fisher
709 Scientific), vancomycin (Acros Organics), bacitracin (Oxoid), bacitracin (Oxoid), ciprofloxacin
710 (Oxoid), and polymyxin b (MP Biomedicals). Detergents tested were: sodium dodecyl sulfate
711 (SDS; anionic; Fisher Scientific), Triton X-100 (nonionic; Acros Organics), cetyltrimethyl
712 ammonium bromide (CTAB; cationic; MP Biomedicals), 3-cholamidopropyl dimethylammonio
713 1-propanesulfonate (CHAPS; zwitterionic; Thermo Scientific). In addition, sensitivity to ethidium
714 bromide (Thermo Scientific) and lysozyme (Thermo Fisher) also was tested. After 48 h, diameters
715 of zones of inhibition around the disks were measured. Experiments were performed in triplicate.
716 For liquid cultures, bacteria were suspended in sMHB, adjusted to OD_{600} 0.4, and 5 ml of each
717 bacterial suspensions was inoculated into 100 ml of either sMHB or sMHB with 5 mM hydrogen

718 peroxide (H_2O_2), 5% sodium chloride (NaCl), or pH 5.5 (pH of sMHB is 6.5). Cultures were
719 grown at 37˚C with rotation at 180 rpm for 24 h with OD_{600} readings recorded every 4 hours.
720
721 *Electron Microscopy*
722 Electron microscopy was used to visualize differences in bacterial envelope structure and cell
723 shape, as previously described (Wu, 2016), with some modifications. LVS, Δ*FTL1678*, FTL1678
724 *trans*-complement, and the Pgp2 *trans*-complement were grown overnight in sMHB, approx.
725 $1x10^9$ CFU of each bacterial strain was pelleted by centrifugation at 7000 × *g* at 4˚C, washed three
726 times in PBS, fixed in 3% (vol/vol) glutaraldehyde (Electron Microscopy Sciences [EMS]) for
727 approx. 24 hours, washed twice in sodium cacodylate buffer (pH 7.4; EMS) for 10 min, suspended
728 in 1% (wt/vol) osmium tetroxide (EMS) in s-collidine buffer (pH 7.4; EMS) for 45 min at room
729 temperature (r/t) to stain and fix the samples, washed two times with sodium cacodylate buffer for
730 10 min each, and tertiary fixation was performed using an aqueous saturated solution of uranyl
731 acetate (pH 3.3; EMS) for 45 min at r/t. Samples were then dehydrated at room temperature using
732 a series of ethanol washes: two washes with 30% ethanol for 10 min each; two washes with 50%
733 ethanol for 10 min each; two washes with 95% ethanol for 10 min each; two washes with 100%
734 ethanol for 10 min each; and two washes with 100% acetone for 10 min each. Samples were then
735 infiltrated with 50% acetone and 50% embedding media (Hard Plus Resin 812, EMS) for 8 h to
736 overnight at r/t. Samples were embedded in 100% embedding media (EMS) and allowed to
737 polymerize for 8 h to overnight at 85°C, then sectioned at 85–90 nm, and visualized using a Tecnai
738 G2 Spirit transmission electron microscope (FEI) at 80 kV and Radius 1.3 (Olympus) camera
739 software at the University of Toledo Electron Microscopy Facility. Experiments were performed
740 twice to confirm reproducibility, with two bacterial preparations fixed, stained, embedded,
741 sectioned, and visualized per experiment.

742

743 *Spheroplasting and Sucrose Density Gradient Centrifugation*

744 Spheroplasting, osmotic lysis, and sucrose density gradient centrifugation was performed as
745 previously described (Huntley, 2007) to determine subcellular localization of FTL1678. Briefly,
746 the histidine-tagged FTL1678 *trans*-complement was grown in sMHB to an OD_{600} of 0.3-0.4,
747 pelleted at 7500 × *g* for 30 min at 10˚C, supernatants were removed, pellets were resuspended in
748 0.75 M sucrose (in 5 mM Tris, pH 7.5) with gentle mixing, 10 mM EDTA (in 5 mM Tris, pH 7.8)
749 was slowly added over 10 min, and the suspension was incubated for 30 min at r/t. After
750 incubation, lysozyme was slowly added to a final concentration of 200 µg ml^{-1}, incubated for 30
751 min at r/t, bacteria were osmotically lysed by dilution into 4.5 × volume of molecular-grade water
752 (Corning) over 11 min with gentle mixing, and incubated for 30 min at r/t. Lysates were
753 centrifuged at 7,500 × *g* for 30 min at 10°C to remove intact cells and cellular debris. Supernatants
754 were collected and centrifuged at 182,500 × *g* for 2 h at 4°C in a F37L 8 × 100 Fiberlite
755 Ultracentrifuge rotor. Following centrifugation, supernatants were removed, membrane pellets
756 were gently resuspended in 6 ml of resuspension buffer (25% [wt/wt] sucrose, 5 mM Tris, 30 mM
757 $MgCl_2$, 1 tablet of Pierce Protease Inhibitor Mini Tablets, EDTA-Free [Thermo Scientific], 5 U
758 Benzonase [Novagen]), suspensions were incubated with gentle mixing for 30 min at room
759 temperature to degrade DNA, and a DC protein assay (Bio-Rad) was performed to determine total
760 protein yield. Linear sucrose gradients were prepared by layering 1.8 ml each of sucrose solutions
761 (wt/wt; prepared in 5 mM EDTA, pH 7.5) into 14- by 95-mm ultracentrifuge tubes (Beckman) in
762 the following order: 55%, 50%, 45%, 40%, 35%, and 30%. Membrane suspensions were layered
763 on top of each sucrose gradient, with less than 1.5 mg of protein per gradient. Sucrose gradients
764 were centrifuged in an SW40 swinging bucket rotor (Beckman) at 256,000 × *g* for 17 h at 4°C.
765 After centrifugation, 500 µl fractions were collected from each gradient by puncturing the bottom

766 of each tube and allowing fractions to drip into microcentrifuge tubes. The refractive index of
767 each fraction was determined using a refractometer (Fisher Scientific) and correlated with a
768 specific density in g ml^{-1} (Price, 1982) to identify outer membrane (OM; 1.17-1.20 g/ml) and inner
769 membrane (IM; 1.13-1.14 g/ml) fractions. Sucrose gradient fractions were examined by
770 immunoblotting as described below.
771
772 *Immunoblotting*
773 Whole cell lysates of FTL1678 *trans*-complement were prepared by suspending bacteria (pelleted
774 at 7000 × *g*) in molecular biology grade water, diluting with SDS-PAGE loading buffer, and
775 boiling for 10 min. Whole cell lysates, OM fractions, IM fractions, and molecular mass standards
776 (Precision Plus protein all blue prestained protein standards; BioRad Laboratories) were separated
777 on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and blots were incubated overnight
778 in blot block (0.1% (vol/vol) Tween 20 and 2% (wt/vol) bovine serum albumin in PBS) at 4°C.
779 Immunoblotting was performed using rat polyclonal antiserum specific for either *F. tularensis* OM
780 protein FopA, *F. tularensis* IM protein SecY (Huntley, 2007) or the Penta-His HRP conjugate
781 antibody (Qiagen).
782
783 *Infections of Mouse Bone Marrow Derived Macrophages (mBMDMs) and J774A.1 cells*
784 Macrophage culture (37˚C with 5% CO_2 unless otherwise indicated) and infections were
785 performed as previously described (Wu, 2016), with some modifications. Bone marrow
786 macrophages were harvested from female C3H/HeN mice. Mice were euthanized by CO_2
787 asphyxiation and cervical dislocation. Femurs and tibias of both hind legs were aseptically-
788 harvested, marrow was flushed from each bone with RPMI-1640 (Hyclone) containing 10% heat-
789 inactivated fetal bovine serum ([HI-FBS], Atlanta Biologicals) and 30% supernatants from day 7

790 L929 cultures (ATCC). Bone marrow was disrupted by repeated passage through a 23-gauge
791 needle and cultured for 4 days. Next, cell media was removed and replaced with RPMI containing
792 10% HI-FBS and 30% supernatant from day 14 L929 cultures, and cells were cultured for 2 days.
793 Approx. 24 h before infection, media was removed, cells were harvested by scraping and
794 centrifugation at 400 × *g* for 10 min at 10˚C, cells were enumerated using a hemocytometer, and
795 diluted to $1x10^5$ cells in RPMI containing 10% HI-FBS. J774A.1 cells (ATCC) were cultured in
796 Dulbecco's Modified Eagle Medium ([DMEM], Gibco) containing 10% HI-FBS. Approx. 24 h
797 before infection, cells were harvested as described above, seeded into individual wells of 24-well
798 plates (Corning) at a concentration of $1x10^5$ cells/well, and incubated overnight. mBMDMs and
799 J774A.1 cells were infected with a multiplicity of infection (MOI) of 100 bacteria to 1 cell (100:1).
800 Following infection, cells were centrifuged at 1,000 × *g* for 10 min at 4˚C, incubated at 37˚C with
801 5% CO_2 for 1 h, washed 1 × with RPMI (or DMEM), media containing 100 μg ml^{-1} gentamicin
802 was added to kill extracellular bacteria, cells were incubated at 37˚C with 5% CO_2 for 1 h, washed
803 1x with RPMI (or DMEM), lysed with 1% saponin for 4 min, serially diluted in PBS, plated onto
804 sMHA plates, and bacteria were enumerated (entry) after 48h. Alternatively, after gentamicin
805 treatment and washing, RPMI (or DMEM) containing 10% HI-FBS was added to cells and they
806 were incubated for 6 or 24 h, lysed, serially-diluted, and plated to determine bacterial numbers.
807

808 *Expression and Purification of Recombinant FTL1678 and FTT0101*

809 *F. tularensis* LVS and SchuS4 genomic DNA were extracted using phenol/chloroform/isoamyl
810 alcohol (Fisher Bioreagents). *FTL1678* and *FTT0101*, without signal sequences (amino acid
811 residues 1-29), were PCR-amplified from LVS and SchuS4 genomic DNA, respectively, using
812 High Fidelity Platinum Taq Polymerase (Life Technologies), and primers 5'FTL1678_BamHI and
813 3'FTL1678_XhoI and 5'FTT0101_BamHI and 3'FTT0101_XhoI, respectively (Table S7).

814 Amplicons and pPROEX HTb were double-digested with BamHI and XhoI, ligated using T4 DNA
815 ligase, and transformed into NEB 10-β *E. coli*. Plasmids were purified using the Qiagen QIAprep
816 Spin Miniprep kit and diagnostic PCR was performed to confirm presence and correct size of the
817 insert. DNA sequencing was performed to confirm insert integrity and plasmid constructs were
818 transformed into Rosetta DE3 *E. coli* (Millipore) for protein expression. Recombinant proteins
819 were expressed and purified as previously described (Ren, 2014) with some modifications.
820 Bacteria were grown in LB-amp to an OD_{600} of 0.4, protein expression was induced for 2 h by the
821 addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 100 mM,
822 bacteria were pelleted by centrifugation, and frozen overnight at −80°C to aid in lysis. Cell pellets
823 were suspended in 10 mM Tris, 500 mM NaCl, and 10 mM imidazole, pH 8.0, sonicated on ice
824 for 10 min with 30 sec intervals, insoluble material was removed by centrifugation at 8,000 × *g*,
825 and supernatants were collected for affinity purification over pre-equilibrated Ni-nitrilotriacetic
826 acid (Ni-NTA) agarose (Qiagen) columns. Eluted recombinant proteins were concentrated in
827 Amicon Ultra-4 centrifugal filter units with 30-kDa cutoff (Millipore), concentrations were
828 determined using the DC BCA protein assay (BioRad), and purity was assessed by SDS-PAGE
829 and Imperial protein staining (Thermo Scientific). An empty vector construct also was expressed
830 and purified as a control in enzymatic assays.
831
832 *Enzymatic Assays for FTL1678/FTT0101 Activity*
833 FTL1678 and FTT0101 recombinant protein activity toward various PG-related compounds were
834 tested in 50 µl reaction mixtures containing 50 mM Tris-HCl, pH 8.0, 0.1 mM substrate, and
835 partially purified enzyme stock (10 µl in 1 M NaCl, 10 mM Tris, pH 8.0). Mixtures were incubated
836 for 30 min to 2 h at 37°C and reactions were stopped by freezing. Substrate and reaction products
837 were separated by HPLC on an ODS-Hypersil 3 µm particle-size C18 column (250 by 4.6 mm;

838 Thermo Scientific). Elutions were performed with 50 mM sodium phosphate buffer, pH 4.5, with
839 or without application of a linear gradient of methanol (from 0 to 50% in 50 min), at a flow rate of
840 0.5 ml/min. Peaks were detected by measuring the absorbance at 207 nm or at 262 nm for UDP-
841 containing nucleotide precursors. Identification of compounds was based on their retention times,
842 compared to authentic standards, as well as on their amino acid and amino sugar composition,
843 determined with a Hitachi model L8800 analyzer (Sciencetec) after hydrolysis of samples in 6 M
844 HCl for 16 h at 95°C. Enzyme activity was calculated by integration of peaks corresponding to
845 substrate and product. Amounts of alanine released by the L,D-carboxypeptidase activity also
846 were determined using an amino acid analyzer. Depending on the substrate used, the amount of
847 partially purified protein varied from 0.9 to 5 µg per assay and incubation times varied from 30
848 min to 4 h. To ensure linearity, substrate consumption was < 20% in all cases. Values represent
849 the means for three independent experiments; the standard deviation was < 10% in all cases.
850 Specific activities were calculated from the amounts of D-Ala (tetrapeptide substrates) or D-Ala-
851 D-Ala (pentapeptide substrates) released during the reaction. FTL1678 double and triple active
852 site mutants were tested in 50 µl reaction mixtures containing 50 mM Tris-HCl, pH 8.0, 0.05 mM
853 substrate, and partially purified enzyme stock (5-10 µl in 1 M NaCl, 10 mM Tris, pH 8.0).
854 Reactions were incubated for 18 hours then analyzed by HPLC as described above. Peaks were
855 detected by measuring absorbance at 207 nm.
856
857 *Peptidoglycan Precursors and Muropeptides*
858 UDP-MurNAc-pentapeptide precursors containing either *meso*-diaminopimelic acid (A_2pm) or L-
859 lysine were prepared by enzymatic synthesis using purified Mur ligases, and UDP-MurNAc-
860 tetrapeptides were generated by treatment of the UDP-MurNAc-pentapeptide precursors with
861 purified *E. coli* PBP5 DD-carboxypeptidase as previously described (Herve, 2007). MurNAc-

862 peptides were obtained by mild acid hydrolysis of UDP-MurNAc-peptides (0.1 M HCl, 100°C, 15
863 min) and were not reduced and thus purified as a mixture of the two α and β anomers (Blanot,
864 1983). Free peptides were prepared by cleavage of MurNAc-peptides with *E. coli* AmiD *N*-
865 acetylmuramoyl-L-alanine amidase (Pennartz, 2009). The *E. coli* peptidoglycan polymer was
866 purified from a Δ*lpp* mutant strain that does not express the Lpp lipoprotein (Leulier, 2003).
867 GlcNAc-1,6-anhydro-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala (TCT) and its dimer (two
868 cross-linked TCT monomers) were produced by digestion of peptidoglycan with *E. coli* SltY lytic
869 transglycosylase and the non-anhydro forms of these monomer and dimer were generated by
870 digestion of the polymer with mutanolysin (Stenbak, 2004). All these compounds were HPLC-
871 purified and their composition was controlled by amino acid and amino sugar content analysis
872 and/or by MALDI-TOF mass spectrometry.
873
874 *Statistics*
875 GraphPad Prism6 was used in various statistical analyses, including: differences in antibiotic,
876 detergent, dye, or lysozyme susceptibility were calculated by one-way ANOVA with multiple
877 comparisons and the Holm-Sidak post-hoc test; differences in EM measurements were determined
878 by unpaired t-tests; differences in median time-to-death and percent survival following *F.*
879 *tularensis* infection of mice were calculated using the log-rank Mantel-Cox test; differences in
880 pathology scores of *F. tularensis*-infected tissues were calculated by two-way ANOVA with
881 multiple comparisons and a Tukey post-hoc test. Differences in lung, liver, spleen, and blood
882 bacterial burdens from infected mice were calculated by one-way ANOVA with multiple
883 comparisons using R software.
884
885

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892

893

894 **Data Availability Statement**

895 Data that support the findings of this study are available in the supplementary material of this
896 article.

897

898

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1115

1116 **Table 1.** Specific Activity and Substrate Specificity of FTL1678 and FTT0101 Enzymes

Substrate	Specific Activity (nmol/min/mg of protein) [a]	
	FTL1678	FTT0101
A_2pm-containing substrates		
GlcNAc-anhydroMurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala (TCT)	21.5	29.0
GlcNAc-MurNAc-L-Ala-γ-D-Glu-*meso*- A_2pm-D-Ala (PG monomer)	15.6	24.2
TCT dimer	7.7	11.8
MurNAc-L-Ala-γ-D-Glu-*meso*-A2pm-D-Ala	5.8	7.8
UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala	4.6	6.3
L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala (free tetrapeptide)	3.4	8.0
MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (pentapeptide)	9.8	6.2
UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (pentapeptide)	5.9	6.4
L-Lysine-containing substrates		
MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala	1.3	2.1
L-Ala-γ-D-Glu-L-Lys-D-Ala	0.7	2.1
UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala	1.2	1.7
Other		
GlcNAc-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm(NH_2)-D-Ala		Very low (<0.5)
GlcNAc-MurNAc-L-Ala-γ-D-Glu(NH_2)-*meso*-A_2pm-D-Ala		Not detected [b]
GlcNAc-MurNAc-L-Ala-γ-D-Glu(NH_2)-*meso*-A_2pm(NH_2)-D-Ala		Not detected [b]
Peptidoglycan polymer		Not detected [b]

1117

1118 [a] Standard enzyme assay conditions are described in the Materials and Methods.

1119 [b] Not detected indicates that no release of alanine was detected.

1120 **Table 2.** Sensitivity of WT and Δ*FTL1678* to Antibiotics, Detergents, and Dyes

Compound	Concentration (µg/disk)	Average Zone of Inhibition, mm (mean ± SD)		
		WT	**Δ*FTL1678***	
Gentamicin	4	2.50 ± 0.10	0.93 ± 0.15	(R)
Tetracycline	5	1.83 ± 0.06	0.80 ± 0.00	(R)
Chloramphenicol	5	3.37 ± 0.12	1.00 ± 0.00	(R)
Ciprofloxacin	5	4.73 ± 0.23	1.90 ± 0.10	(R)
Ampicillin	200	2.57 ± 0.12	3.53 ± 0.15	(S)
Vancomycin	20	1.27 ± 0.21	2.60 ± 0.10	(S)
Bacitracin	182	1.10 ± 0.10	1.20 ± 0.10	
Polymyxin B	100	0.70 ± 0.00	0.70 ± 0.00	
Lysozyme	1000	0.70 ± 0.00	1.67 ± 0.06	(S)
Ethidium Bromide	5	2.73 ± 0.15	0.80 ± 0.00	(R)
Triton-X100	750	3.40 ± 0.27	2.63 ± 0.06	(R)
SDS	1000	1.20 ± 0.10	1.80 ± 0.00	(S)
CTAB	50	0.73 ± 0.06	0.80 ± 0.10	
CHAPS	50	0.70 ± 0.00	0.70 ± 0.00	

1122

1123 [a] (R) indicates Δ*FTL1678* is significantly more resistant than WT by one-way ANOVA ($P<0.05$)

1124 [a] (S) indicates Δ*FTL1678* is significantly more sensitive than WT by one-way ANOVA ($P<0.05$)

1125 **Figure 1. Amino acid alignment of bacterial L,D-carboxypeptidases.** Clustal Omega amino
1126 acid alignment of *E. coli* LdcA (BAA36050.1), *P. aeruginosa* LdcA (Q9HTZ1), *N. gonorrhoeae*
1127 LdcA (YP_208343.1), *F. tularensis* Type B FTL1678, and *F. tularensis* Type A FTT0101, and *C.*
1128 *jejuni* Pgp2 (WP_002856863). Percent identities (pid), compared to *E. coli* LdcA, are indicated.
1129 Black shading indicates similar residues. Red shading indicates the catalytic triad.
1130
1131 **Figure 2. FTL1678 is OM-associated.** Spheroplasting, osmotic lysis, and sucrose density
1132 gradient centrifugation were performed to separate inner membranes (IM) and outer membranes
1133 (OM) from *F. tularensis* ΔFTL1678 *trans*-complemented with a 6× histidine-tagged FTL1678.
1134 Whole-cell lysates (WCL), OM fractions, and IM fractions were separated by SDS-PAGE,
1135 transferred to nitrocellulose, and immunoblotting was performed using antisera specific for the
1136 OM control protein FopA (αFopA), IM control protein SecY (αSecY), or histidine-tagged
1137 FTL1678.
1138
1139 **Figure 3. FTL1678 controls bacterial morphology.** Electron micrograph images of: (A) Wild-
1140 type LVS or (B) Δ*FTL1678* grown in sMHB to OD_{600} of 0.4. Scale bars represent 100 nm; (C)
1141 Outer membrane thickness measurements (nm) were measured for WT and Δ*FTL1678*, n=50; (D)
1142 Cell width measurements (nm) for WT and Δ*FTL1678*, n=175. **** indicates $P<0.0001$.
1143
1144 **Figure 4. Deletion of *FTL1678* increases resistance to stressors.** WT and Δ*FTL1678* were
1145 grown in 75 ml sMHB at: (A) 37˚C, (B) sMHB at 40˚C, (C) sMHB + 60 μM $CuCl_2$, (D) sMHB +
1146 5 mM H_2O_2, (E) sMHB + 5% NaCl, or (F) sMHB at pH 5.5. Bacteria were grown for 24 hours
1147 and OD_{600} measurements were recorded every 4 hours.

1148 **Figure 5. ΔFTL1678 is fully-attenuated in a mouse pulmonary infection model.** (A) Groups
1149 of 5 C3H/HeN mice were intranasally-infected with 10^5 CFU of either wild-type WT, Δ*FTL1678*,
1150 or Δ*FTL1678 trans*-complemented with *FTL1678* [FTL1678 compl]. Animal health was
1151 monitored daily through day 21 post-infection. **** indicates $P<0.0001$; (B) Lungs, livers,
1152 spleens, and blood were aseptically harvested from mice infected with 10^4 CFU of either WT or
1153 Δ*FTL1678* on days 2 and 5 post-infection and plated to enumerate bacterial numbers. * indicates
1154 $P<0.01$; (C) Groups of 5 C3H/HeN mice were intranasally-infected with 10^5 CFU of either LVS,
1155 Δ*FTL1678*, FTL1678 *trans*-complement [FTL1678 compl], or *C. jejuni* Pgp2 *trans*-complement
1156 [Pgp2 compl]. Animal health was monitored through day 21 post-infection. ** indicates $P<0.01$.
1157

1158 **Figure 6. FTL1678 is required for *F. tularensis* replication in macrophages.** (A) J774A.1
1159 macrophages or mouse bone marrow-derived macrophages (mBMDMs) were infected with WT
1160 or Δ*FTL1678* at an MOI of 100:1 and bacterial numbers were enumerated at entry (0 h), 6 h, and
1161 24 h post-infection. (B) Fold change in bacterial numbers from 6 to 24 h post-infection was
1162 calculated. * indicates $P< 0.01$.
1163

1164 **Figure 7. Δ*FTL1678* protects against fully-virulent Type A *F. tularensis* SchuS4.** (A) Groups
1165 of 5 C3H/HeN mice were intranasally infected with either 10^5 CFU WT or 10^5, 10^7, or 10^9 CFU
1166 Δ*FTL1678*. On day 29 post-infection, mice were boosted with 10^9 CFU Δ*FTL1678* and animal
1167 health was monitored daily through day 50 post-infection. *** $P<0.001$; (B) Mice from A were
1168 intranasally-challenged with 120 CFU of wild-type SchuS4 [BSL3; 6× LD50]. Animal health was
1169 monitored daily through day 26 post-infection. * indicates $P<0.001$.
1170

1171 **Figure 8. Δ*FTL1678* does not induce tissue damage.** (A) Hematoxylin and eosin (H&E)-
1172 stained lungs, livers, and spleens were examined from either uninfected, *F. tularensis* WT LVS-,
1173 or Δ*FTL1678*-infected mice at 10× objective. (B) Tissues were graded on a scale of 0 to 4, with 4
1174 being the most severe. * indicates $P<0.05$.
1175
1176 **Figure 9. Model of *F. tularensis* peptidoglycan synthesis and recycling pathways.**
1177 Bioinformatic analyses were used to predict proteins that may be involved in peptidoglycan
1178 synthesis and recycling in *F. tularensis*. *F. tularensis* LVS gene locus tags are indicated, with *E.*
1179 *coli* or Gram-negative ortholog protein names. OM, outer membrane. IM, inner membrane.
1180 GlcNAc, N-Acetylglucosamine. MurNAc, N-Acetylmuramic acid. Pal, OM-localized
1181 peptidoglycan-associated lipoprotein. TolB, periplasmic protein that interacts with Pal and
1182 peptidoglycan. HMM PBP, high molecular weight penicillin binding protein. LMM PBP, low
1183 molecular weight penicillin binding protein.
1184

1185 **Supporting Information**

1186

1187 **Table S1.** Bioinformatic analyses of FTL1678 localization

1188

1189 **Table S2.** Endopeptidase activity of FTL1678

1190

1191 **Table S3.** Sensitivity of WT *F. tularensis* SchuS4 and Δ*FTT0101* to antibiotics, detergents, and

1192 dyes

1193

1194 **Table S4.** Specific activity of FTL1678, FTL1678 double mutants, and FTL1678 triple mutants

1195 to the TCT monomer

1196

1197 **Table S5.** Sensitivity of WT LVS, ΔFTL1678, FTL1678 trans-complement, and Pgp2 trans-

1198 complement to antibiotics, detergents, and dyes

1199

1200 **Table S6.** Bacterial strains and plasmids used in this study

1201

1202 **Table S7.** Primers used in this study

1203

1204 **Figure S1. FTL1678 contains a putative L,D-carboxypeptidase domain.** NCBI Conserved

1205 Domain search results for *F. tularensis* FTL1678.

1206

1207 **Figure S2. F. tularensis TolB is OM-localized.** Spheroplasting, osmotic lysis, and sucrose

1208 density gradient centrifugation were performed to separate inner membranes (IM) and outer

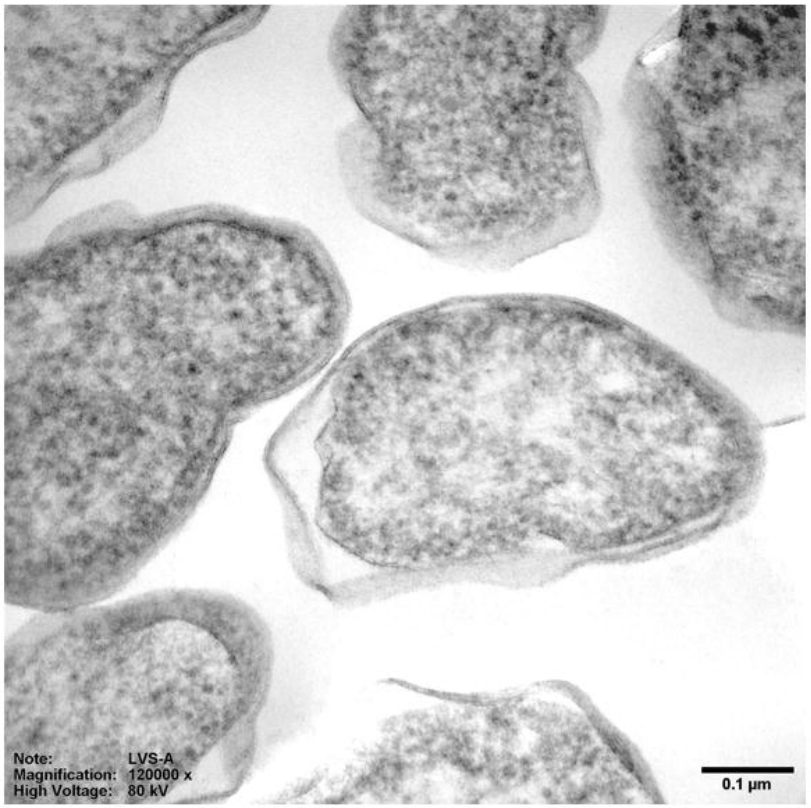
1209 membranes (OM) from *F. tularensis* Δ*FTL1678 trans*-complemented with a 6x histidine-tagged
1210 FTL1678. Whole-cell lysates (WCL), OM fractions, and IM fractions were separated by SDS-
1211 PAGE, transferred to nitrocellulose, and immunoblotting was performed using antisera specific
1212 for the periplasmic protein TolB (αTolB).
1213
1214 **Figure S3. ΔFTL1678 does not have a growth defect.** WT and ΔFTL1678 were grown in
1215 sMHB for 24 hours at 37˚C. Samples were taken every 4 hours for (A) OD600 measurements
1216 and (B) CFU enumeration following serial-dilution and plating.
1217
1218 **Figure S4. ΔFTL1678 has septation defects.** Transmission electron micrograph images of
1219 ΔFTL1678 showing aberrant septal formation and reduced ability to separate cells. Images taken
1220 at: (A) 49,000×, scale bar represents 200 nm; and (B) 6,800×, scale bar represents 2 µm. In
1221 (A), red arrows point to formed septa that have not separated in ΔFTL1678 and white arrows
1222 point to new septa that are forming in ΔFTL1678. Experiments were performed twice to confirm
1223 reproducibility, with two bacterial preparations fixed, stained, embedded, sectioned, and
1224 visualized per experiment. Representative images shown.
1225
1226 **Figure S5. FTT0101 is not required for *F. tularensis* Type A strain SchuS4 virulence.**
1227 C3H/HeN mice were intranasally infected with either 80 CFU SchuS4 (n=3 mice) or 12 CFU
1228 Δ*FTT0101* (n=5 mice).
1229
1230 **Figure S6. LdcA catalytic triad is not essential for *F. tularensis* virulence.** Groups of 5
1231 C3H/HeN mice were intranasally-infected with 10^5 CFU of either *F. tularensis* WT LVS,
1232 Δ*FTL1678*, Δ*FTL1678 trans*-complemented with FTL1678 (FTL1678 compl), Δ*FTL1678 trans*-

1233 complemented with S134A (S134A), Δ*FTL1678 trans*-complemented with E239A (E239A), or
1234 Δ*FTL1678 trans*-complemented with H308A (H308A). Animal health was monitored daily
1235 through day 21 post-infection. ** $P<0.01$.
1236
1237 **Figure S7. FTL1678 *trans*-complement and *C. jejuni* Pgp2 *trans*-complement restore *F.***
1238 ***tularensis* phenotype.** Transmission electron micrograph images of: (A) Δ*FTL1678 trans*-
1239 complemented with *FTL1678* [FTL1678 compl] or (B) Δ*FTL1678 trans*-complemented with *C.*
1240 *jejuni pgp2* [Pgp2 compl]. Bacteria were grown in sMHB to OD_{600} of 0.4. Scale bars represent
1241 100 nm. (C) Outer membrane thickness and (D) cell width of FTL1678 compl and Pgp2 compl
1242 were compared to WT LVS and D*FTL1678.* **** indicates $P<0.0001$.
1243
1244 **Figure S8. FTL1678 *trans*-complement and *C. jejuni* Pgp2 *trans*-complement exhibit**
1245 **similar phenotypes to stressors as WT *F. tularensis*.** WT LVS [WT], Δ*FTL1678,* Δ*FTL1678*
1246 *trans*-complemented with *FTL1678* [FTL1678 compl], or Δ*FTL1678 trans*-complemented with
1247 *C. jejuni pgp2* [Pgp2 compl] were grown in either: (A) sMHB; (B) sMHB with 5 mM H_2O_2; (C)
1248 sMHB with 5% NaCl; or (D) sMHB at pH 5.5. Cultures were incubated for 24 hours and OD_{600}
1249 measurements were recorded every 4 hours.

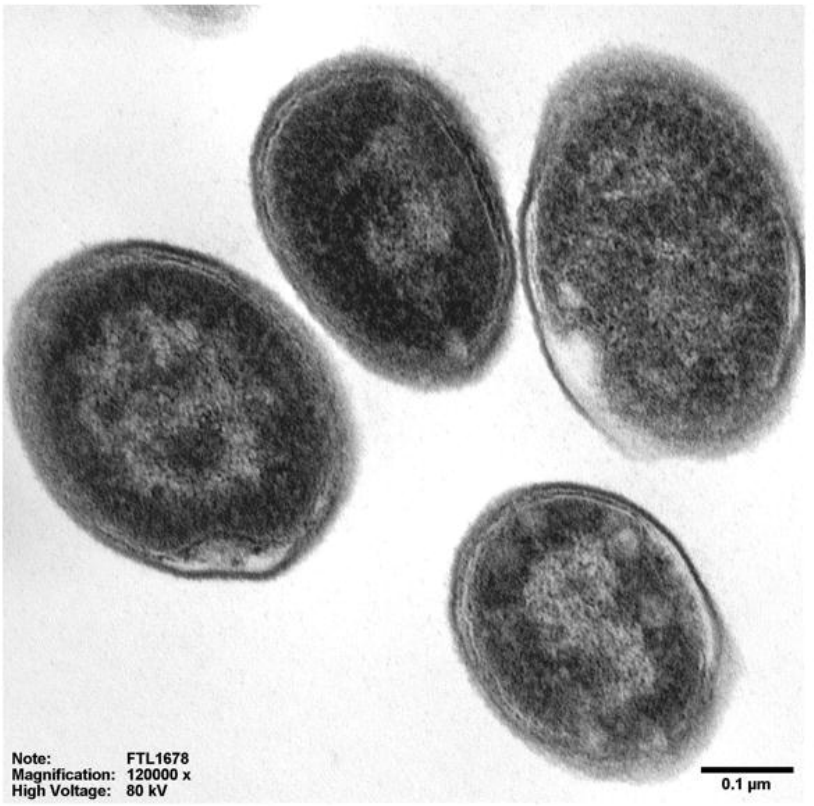
```
            pid   1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 80
Ec LdcA  100.0%   ----------------------------------------------------------MSLFHLIAPSGYCIKQHAALRG
Pa LdcA   23.0%   ----------------------------------------------MTSRPSSDQTWQPIDGRVALIAPASAIATDVLEAT
Ng LdcA   30.3%   MTEPTSRRRFLKTCTAAGAGLLQACGTSATSVPPLPSSHSVVKARTVPLQTPRRQSSDGNLLRVVASSGFAEDTNRVNTA
FTL1678   17.6%   ----------------------------MLLKNNYLVSIILVVLIMIVTKSFACAATDYNKVALINVS-TQYYPNDIKQA
FTT0101   17.6%   ----------------------------MLLKNNYLVSIILVVLIMIVTKSFACAATDYNKVALINVS-TQYYPNDIKQA
Cj Pgp2    6.3%   ----------------------------------MLKRLALLITLSSLMLHASDLVKIYLNQGLDAVGVAIEKELTQKD

            pid  81                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 160
Ec LdcA  100.0%   IQRLTDAGHQVNNVEVIARRCERFAGTETERLEDLNSLAR-LTTPNTIVLAVRGGYG----ASRLLADIDWQALVARQQH
Pa LdcA   23.0%   LRQLEVHGVDYHLGRHVEARYRYLAGTVEQRLEDLHNAFD--MPDITAVWCLRGGYG----CGQLLPGLDWGRLQAASPR
Ng LdcA   30.3%   LTRLYNAGFTVTNQQAGSRRFQRFAGTDAQRAADFQEVASGRVATPKVLMGLRGGYG----AARILPHIDFASLGARMRE
FTL1678   17.6%   EKALKDTGYNTT-YKYLDIYPSDFGYSNPDSIRAKILLDALLDKNIDIIWFLKGGGG----AFNLLPYL-YDHINELKKA
FTT0101   17.6%   EKALKDTGYNTT-YKYLDIYPSDFGYSNPDSIRAKILLDALLDKNIDIIWFLKGGGG----AFNLLPYL-YDHINELKKA
Cj Pgp2    6.3%   FWLSEIGDKNISLGYYDDNVAIVLTNKTDKILRVYSYEDGKIRKDFEQKEIITGLMGDKKIEGDLKTPVGFYELGRKFNP

            pid 161                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 240
Ec LdcA  100.0%   DPLLICGHSDFTAIQCGLLAHGNVITFSGPMLVANFGADELNAFTEHHFWLALRNETFTIEWQ-----------GEGPTC
Pa LdcA   23.0%   P---LIGFSDISVLLSAFFRHGLPAIHGPVATGLGLSPLSAPREQQERLASLASVSRLLAGIDHELP----VQHLGGHKQ
Ng LdcA   30.3%   HGTLFFGFSDVCAVQLALLAKGNMMSFAGPMAYSDFGKPAPGAFTMDAFIKGATQNRLTVDVPY----------IQRADV
FTL1678   17.6%   KPKILVGFSDVTAIHFFVNNVLGWKSLHGVVAAYNKNAYSSQKIEKIRINDLERIPNITEIINNGISYDKLMPMNKMAYN
FTT0101   17.6%   KPKILVGFSDVTAIHFFVNNVLGWKSLHGVVAAYNKNAYSSQKIGKIRINDLERIPNITEIINNGISYDKLMPMNKMAYN
Cj Pgp2    6.3%   GDPYYGPFAFATTYPNLLDKVQGKTGGGIWIHGYPLDGSRLDEFKTRGCIALFNNN-----------------LEKFAQV

            pid 241                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 320
Ec LdcA  100.0%   RAEGTLWGGNLAMLISLIGTP-WMPKIENGILVLEDINEHPFRVERMLLQLYHAGILPRQKAIILGSFSGSTP-NDYDAG
Pa LdcA   23.0%   RVEGALIGGNLTALACMAGTLGGLHAPAGSILVLEDVGEPYYRLERSLWQLLESIDAFQLGAICLGSFTDCPR---KEVA
Ng LdcA   30.3%   ETEGTLWGGNLSVLASLAGTP-YMPDIDGGILFLEDVGEQPYRIERMLNTLYLSGILGKQRAIVFGDFRMEKIRDLYDSS
FTL1678   17.6%   GTDGSIVGGNMTLIYSYFSTV-YQQDISTKILFLEDTGISFRQLDRSLHQLLYLPENKKPEAIIFGQFYPLDP--TDDQR
FTT0101   17.6%   GIDGSIVGGNMTLIYSYFSTV-YQQDISTKILFLEDTGISFRQLDRSLHQLLYLPENKKPEAIIFGQFYPLDP--TDDQR
Cj Pgp2    6.3%   VQDKKVFVMTEEKEKIRAKKDQIASLLADLFTWKLAWTNSDTNTYLSFYDEQEFKRFDKMKFEQFASMKKSIFSRKEDKK

            pid 321                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 400
Ec LdcA  100.0%   YNLESVYAFLRSRLSIPLITGLDFGHEQRTVTLPLGAHA-ILNNTREGTQLTISGHPVLKM-------------------
Pa LdcA   23.0%   HSLERIFGEYAAAIEVPLYHHLPSGHGAQNRAWPYGKTAVLEGNRLRW--------------------------------
Ng LdcA   30.3%   YDFSAVAKHISRTAKIPVLTGFPFGHIADKITFPLGAHTRIRMNGNGGYSVAFEGYPTLDASALTLDTLLPPPDLPIFPE
FTL1678   17.6%   LIYKTVIKKFAKTFNRPVYYFPFIGHGQYNKPLLLGVTSNIKCSKETIFCTLKQK------------------------
FTT0101   17.6%   LIYKTVIKKFAKTFNRPVYYFPFIGHGQYNKPLLLGVTSNIKCSKETIFCTLKQK------------------------
Cj Pgp2    6.3%   IKFSDINISPYPNLENETMYRISFYEDYYTKNYQFRGDKILYVKIDSKGKMKILAEQ----------------------

            pid 401          408
Ec LdcA  100.0%   --------
Pa LdcA   23.0%   --------
Ng LdcA   30.3%   SGVADISE
FTL1678   17.6%   --------
FTT0101   17.6%   --------
Cj Pgp2    6.3%   --------
```

αFopA
WCL
OM
IM
37 kDa
αSecY
WCL
OM
IM
50 kDa
FTL1678
WCL
OM
IM
37 kDa

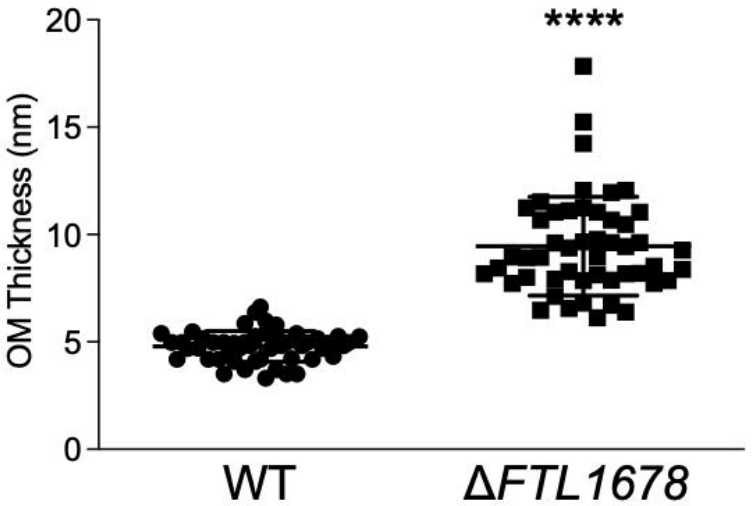
A.


Note: LVS-A
Magnification: 120000 x
High Voltage: 80 kV
0.1 µm

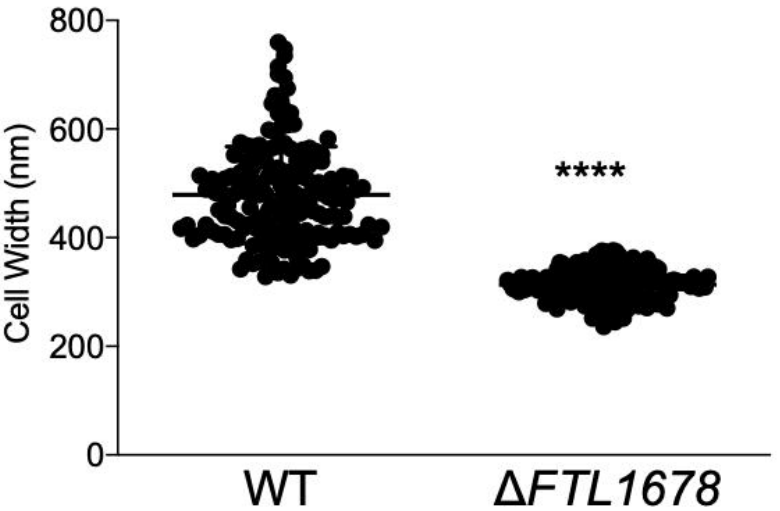
B.

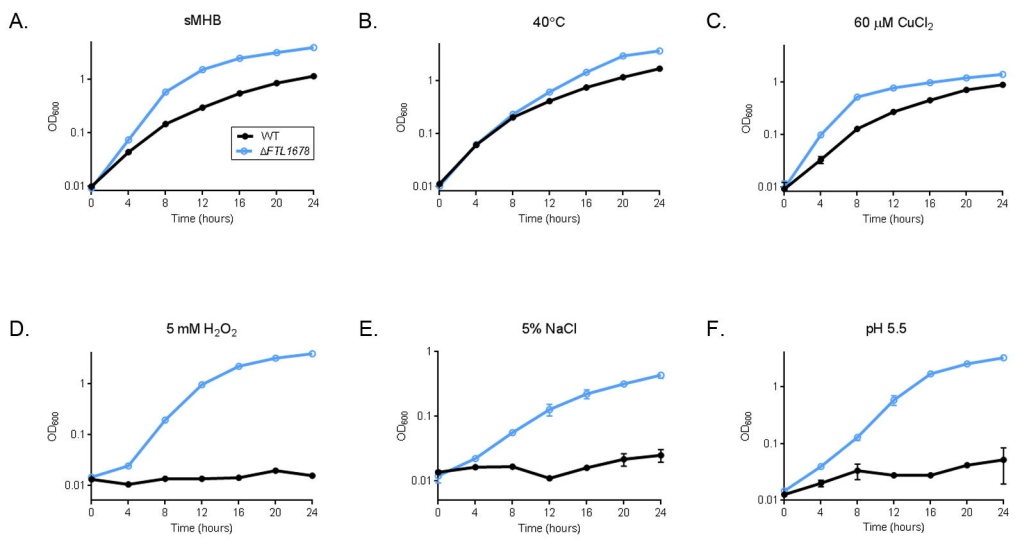

Note: FTL1678
Magnification: 120000 x
High Voltage: 80 kV
0.1 µm

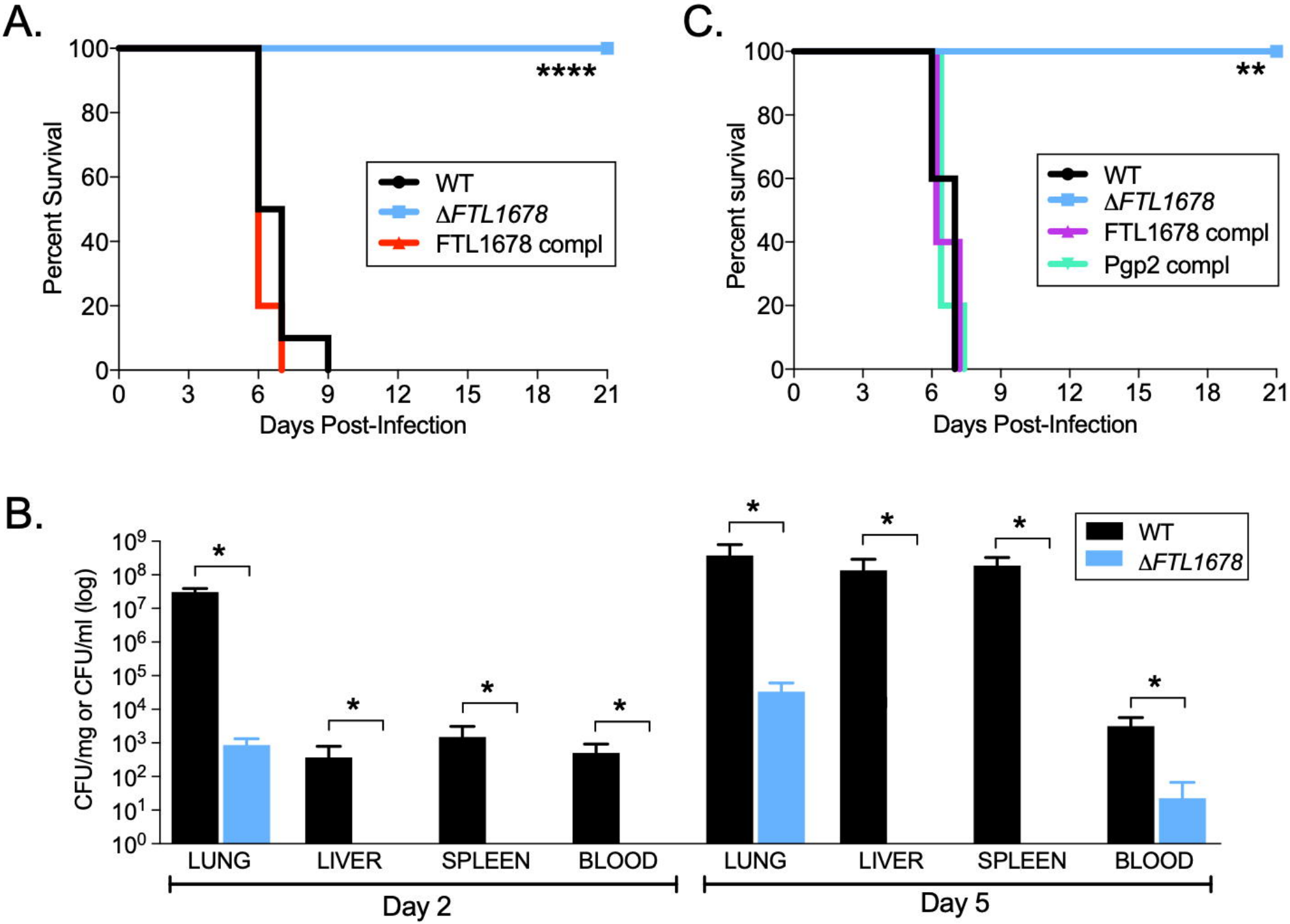
C.

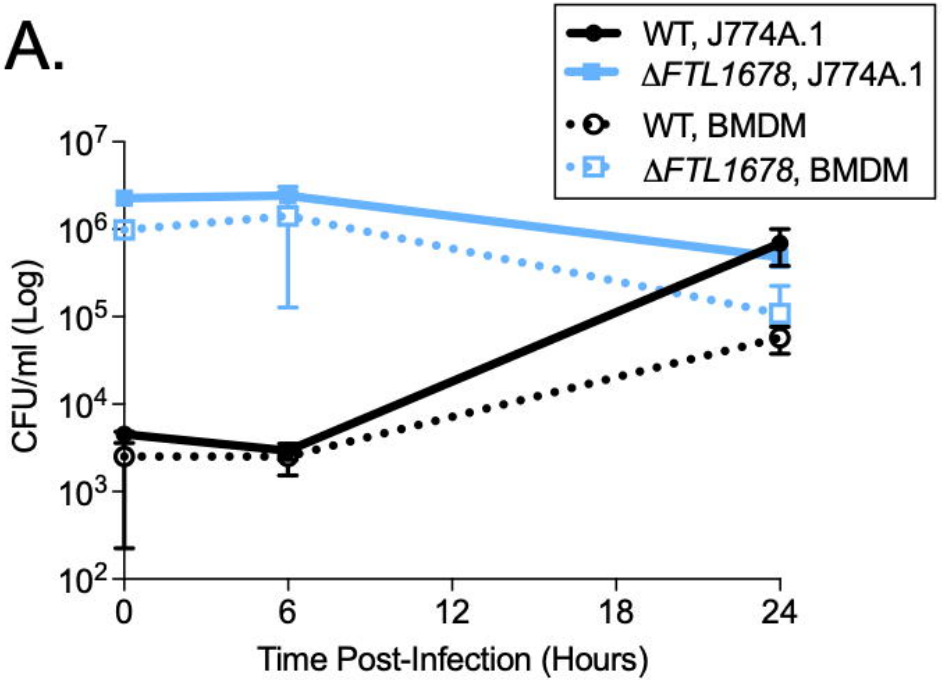

OM Thickness (nm)
20
15
10
5
0
****
WT
ΔFTL1678

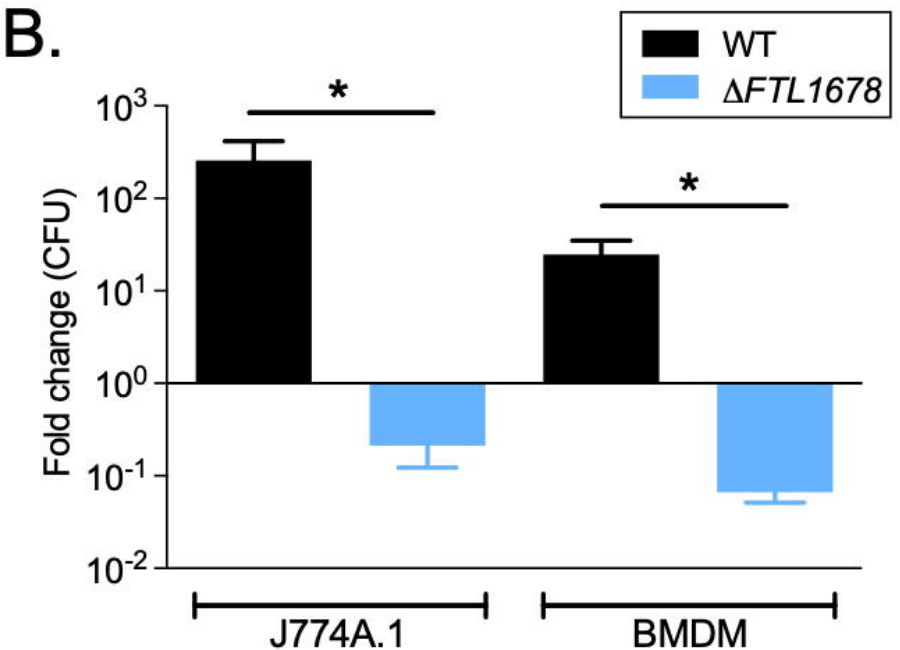
D.

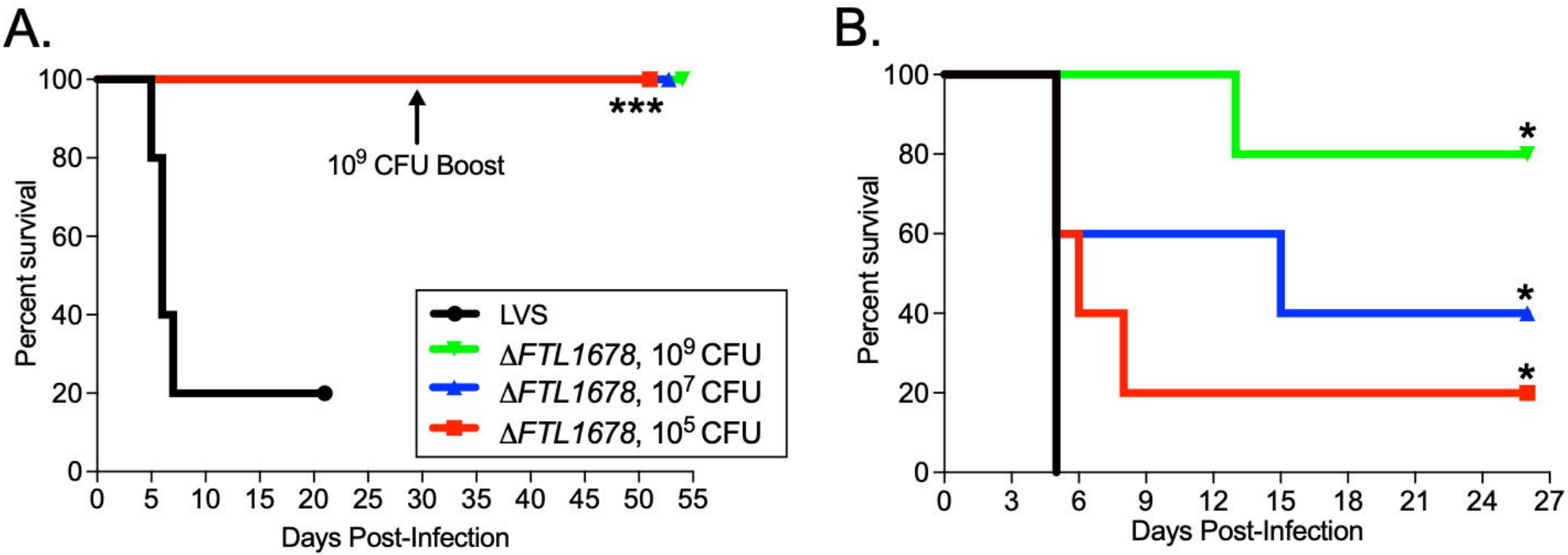

Cell Width (nm)
800
600
400
200
0
****
WT
ΔFTL1678


A.
sMHB
B.
40°C
C.
60 μM CuCl2
D.
5 mM H2O2
E.
5% NaCl
F.
pH 5.5
OD600
Time (hours)
WT
ΔFTL1678

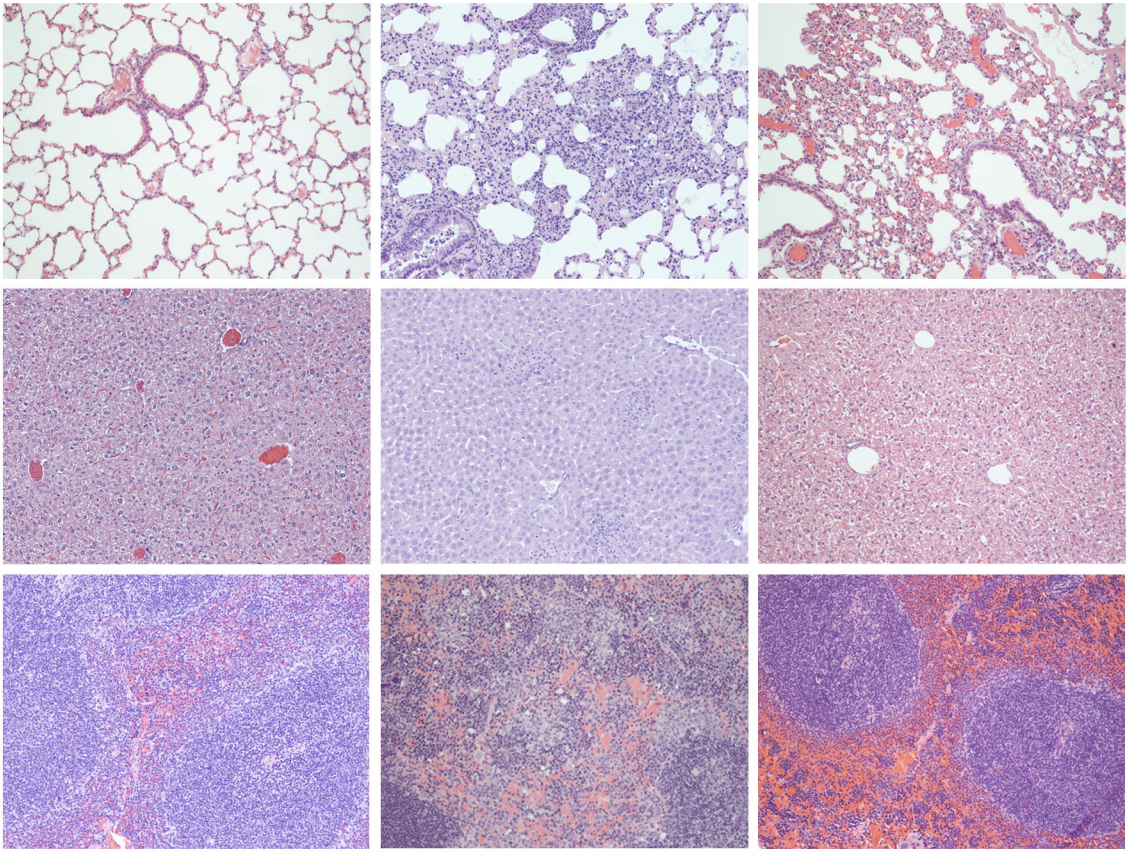

A.
Percent Survival
Days Post-Infection
****
WT
ΔFTL1678
FTL1678 compl
C.
Percent survival
Days Post-Infection
**
WT
ΔFTL1678
FTL1678 compl
Pgp2 compl
B.
CFU/mg or CFU/ml (log)
LUNG
LIVER
SPLEEN
BLOOD
Day 2
Day 5
WT
ΔFTL1678


A.
WT, J774A.1
ΔFTL1678, J774A.1
WT, BMDM
ΔFTL1678, BMDM
CFU/ml (Log)
10^7
10^6
10^5
10^4
10^3
10^2
0
6
12
18
24
Time Post-Infection (Hours)

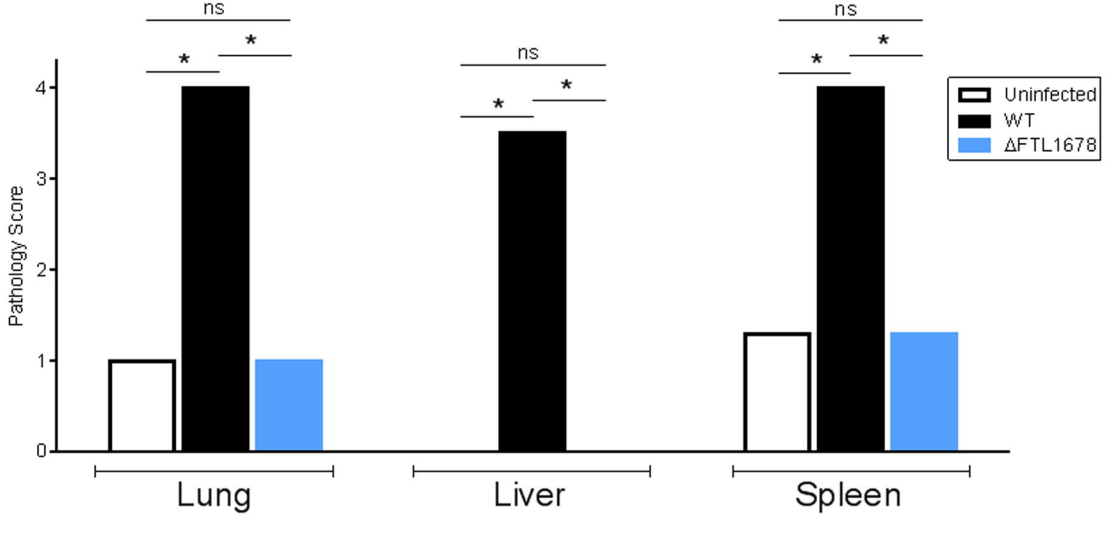

B.
WT
ΔFTL1678
Fold change (CFU)
10^3
10^2
10^1
10^0
10^-1
10^-2
*
*
J774A.1
BMDM

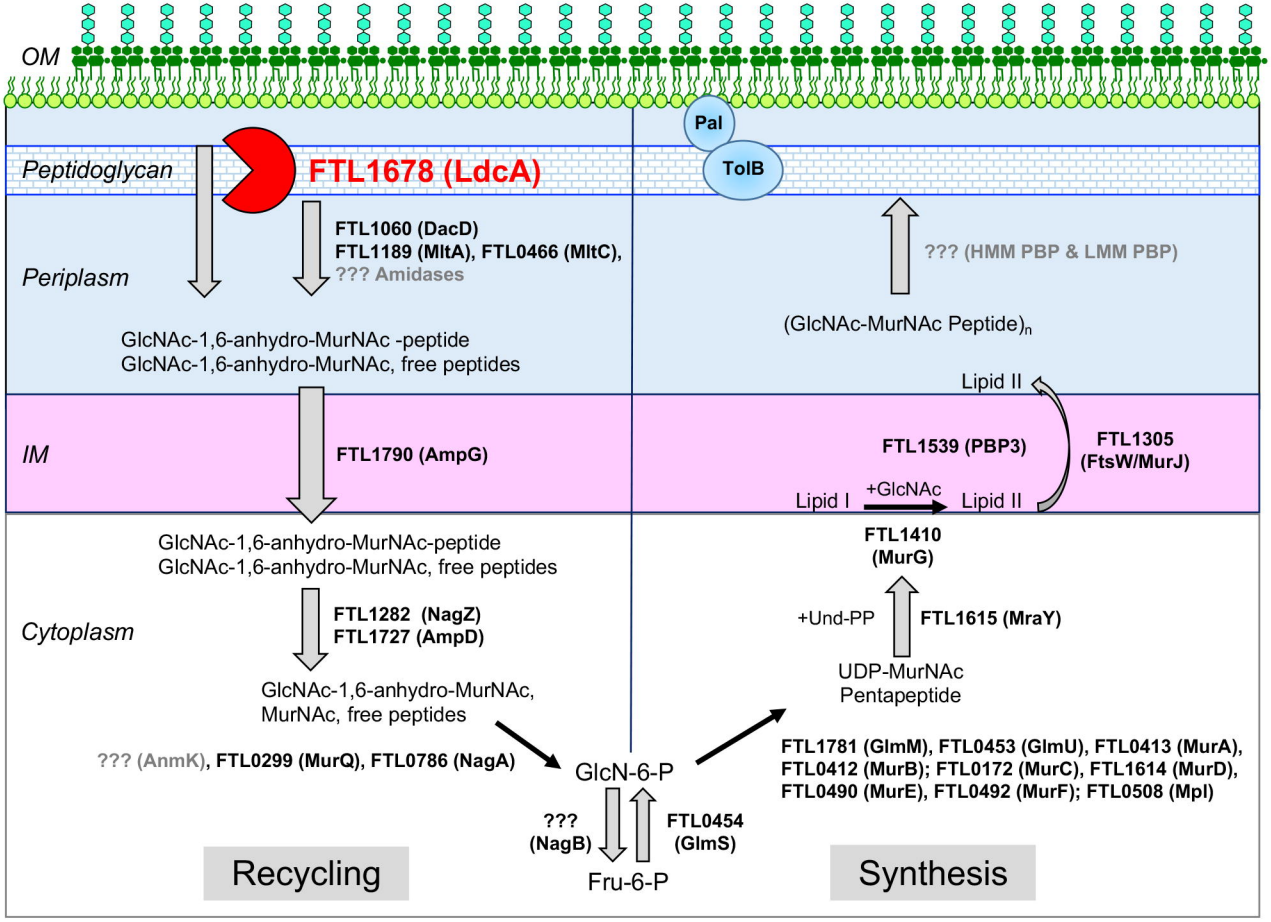

A.
100
80
60
40
20
0
Percent survival
0 5 10 15 20 25 30 35 40 45 50 55
Days Post-Infection
10^9 CFU Boost
***
LVS
ΔFTL1678, 10^9 CFU
ΔFTL1678, 10^7 CFU
ΔFTL1678, 10^5 CFU
B.
100
80
60
40
20
0
Percent survival
0 3 6 9 12 15 18 21 24 27
Days Post-Infection
*
*
*

A.


Uninfected
LVS
ΔFTL1678
Lung
Liver
Spleen

B.


ns
*
*
ns
*
*
ns
*
*
Pathology Score
0
1
2
3
4
Uninfected
WT
ΔFTL1678
Lung
Liver
Spleen


OM
Peptidoglycan
FTL1678 (LdcA)
Pal
TolB
Periplasm
FTL1060 (DacD)
FTL1189 (MltA), FTL0466 (MltC),
??? Amidases
??? (HMM PBP & LMM PBP)
GlcNAc-1,6-anhydro-MurNAc -peptide
GlcNAc-1,6-anhydro-MurNAc, free peptides
(GlcNAc-MurNAc Peptide)n
Lipid II
IM
FTL1790 (AmpG)
FTL1539 (PBP3)
FTL1305
(FtsW/MurJ)
Lipid I
+GlcNAc
Lipid II
GlcNAc-1,6-anhydro-MurNAc-peptide
GlcNAc-1,6-anhydro-MurNAc, free peptides
FTL1410
(MurG)
Cytoplasm
FTL1282 (NagZ)
FTL1727 (AmpD)
+Und-PP
FTL1615 (MraY)
UDP-MurNAc
Pentapeptide
GlcNAc-1,6-anhydro-MurNAc,
MurNAc, free peptides
??? (AnmK), FTL0299 (MurQ), FTL0786 (NagA)
GlcN-6-P
FTL1781 (GlmM), FTL0453 (GlmU), FTL0413 (MurA),
FTL0412 (MurB); FTL0172 (MurC), FTL1614 (MurD),
FTL0490 (MurE), FTL0492 (MurF); FTL0508 (Mpl)
???
(NagB)
FTL0454
(GlmS)
Fru-6-P
Recycling
Synthesis