Francisella tularensis type A Strains Cause the Rapid Encystment of Acanthamoeba castellanii and Survive in Amoebal Cysts for Three Weeks post Infection

S. H. El-Etr, J. Margolis, D. Monack, R. Robison, M. Cohen, E. Moore, A. Rasley

July 30, 2009

Disclaimer

1   ***Francisella tularensis* type A Strains Cause the Rapid Encystment of *Acanthamoeba***

2   ***castellanii* and Survive in Amoebal Cysts for Three Weeks post Infection**

3   Sahar H. El-Etr[1]*, Jeffrey J. Margolis[2], Denise Monack[2], Richard A. Robison[3], Marissa Cohen[3],

4   Emily Moore[3] and Amy Rasley[1]

5

6   Biosciences and Biotechnology Division, Lawrence Livermore National Laboratory, Livermore,

7   CA 94550, USA[1]. Department of Microbiology and Immunology, Stanford University Medical

8   School, Stanford, CA 94305[2], USA. Department of Microbiology and Molecular Biology,

9   Brigham Young University, Provo, UT 84602, USA[3].

10

11

12

13

14

15

16

17

18

19

20

21   * Corresponding Author: Sahar H. El-Etr, Biosciences and Biotechnology Division, Lawrence

22   Livermore National Laboratory, Livermore CA 94550, USA. Tel: (925) 423-0383, Fax: (925)

23   422-2282, email: eletr1@llnl.gov.

ABSTRACT

24

25   *Francisella tularensis,* the causative agent of the zoonotic disease tularemia, has recently

26 gained increased attention due to the emergence of tularemia in geographical areas where the

27 disease has been previously unknown, and the organism's potential as a bioterrorism agent.

28 Although *F. tularensis* has an extremely broad host range, the bacterial reservoir in nature has

29 not been conclusively identified. In this study, the ability of virulent *F. tularensis* strains to

30 survive and replicate in the amoeba *Acanthamoeba castellanii* was explored. We observe that *A.*

31 *castellanii* trophozoites rapidly encyst in response to *F. tularensis* infection and that this rapid

32 encystment phenotype (REP) is caused by factor(s) secreted by amoebae and/or *F. tularensis* into

33 the co-culture media. Further, our results indicate that in contrast to LVS, virulent strains of *F.*

34 *tularensis* can survive in *A. castellanii* cysts for at least 3 weeks post infection and that induction

35 of rapid amoeba encystment is essential for survival. In addition, our data indicate that

36 pathogenic *F. tularensis* strains block lysosomal fusion in *A. castellanii*. Taken together, these

37 data suggest that the interactions between *F. tularensis* strains and amoeba may play a role in the

38 environmental persistence of *F. tularensis*.

39

40

41

42

43

44

45

46

INTRODUCTION

48 *Francisella tularensis* is the etiological agent of the zoonotic disease tularemia, also

49 known as rabbit fever (35, 53). *F. tularensis* strains belonging to subspecies *tularensis* and

50 *holarctica*, which are both prevalent in the northern hemisphere, cause the majority of reported

51 cases of tularemia (36). Subspecies *tularensis* is highly contagious, with an infectious dose of 1-

52 10 bacteria and is associated with more severe disease (21). Though described more than a

53 century ago as a disease common among hunters and trappers, tularemia has recently been

54 reported in areas with no previous known risk (20, 25, 31, 42). *F. tularensis* infects a broad range

55 of wildlife species (36) and a number of arthropods, such as ticks and flies, are known to be

56 vectors (36, 49). Humans are usually infected either through an insect bite or by inhalation of

57 aerosolized bacteria (49). Tularemia can be fatal in up to 30% of untreated cases (36, 49) with

58 the mortality rate reaching 90% in pneumonic infections as described in early studies conducted

59 on vaccinated human volunteers (44-46, 49). Due to its highly infectious nature and its potential

60 for use as a bio-terrorism agent, *F. tularensis* has been classified as a class A biothreat pathogen

61 by the Centers for Disease Control and Prevention (CDC), mandating human tularemia become a

62 reportable disease since 2000 (15, 37). In addition, the absence of a licensed vaccine for

63 prophylaxis (36) makes understanding the virulence mechanisms used by this pathogen

64 imperative for the development of efficacious measures to prevent or treat human disease.

65

66 Though *F. tularensis* has been isolated from more than 250 wildlife species (21), the

67 acute nature of the infections and the resultant high mortality rates in these hosts indicate the

68 bacterial reservoir(s) in nature have yet to be identified. Tularemia outbreaks involving

69 subspecies *holarctica* have often been linked to water sources (6, 40) and a positive PCR field

70 test was reported for *Francisella* during such an outbreak in Norway (5). Abd et al. reported that

71 the *F. tularensis* live vaccine strain (LVS) is able to survive and replicate in the amoeba

72 *Acanthamoeba castellanii* (1), suggesting a potential link between amoeba-*Francisella*

73 interactions and environmental persistence. *A. castellanii*, a free living environmental amoeba, is

74 known to serve as a reservoir for a number of pathogenic microorganisms (24). However, to

75 date, interactions of virulent *F. tularensis* subspecies *tularensis* strains with amoebae have not

76 been documented. The ability of several human intracellular pathogens, including *Legionella*

77 *pneumophila* and *Mycobacterium avium* to infect and survive within amoebae has been well

78 characterized (10, 12). In addition to playing a role in environmental survival and dissemination,

79 growth in *A. castellanii* has been shown to enhance the ability of *L. pneumophila* and *M. avium*

80 to survive and replicate in host macrophages (10, 12) and to enhance the virulence of both

81 species in mice (7, 12). Since *F. tularensis* species are facultative intracellular pathogens that

82 primarily survive in macrophages, probing the *Francisella*-amoeba interaction may provide

83 insights into *Francisella* pathogenesis as well as environmental survival. In this study we

84 investigated the ability of virulent type A strains of *F. tularensis* to survive in *A. castellanii* with

85 a focus on understanding the role of *Francisella*-amoeba interactions in environmental

86 persistence.

87

88

89

90

91

92

MATERIALS AND METHODS

94 **Strains and growth conditions.** *F. tularensis* subsp. *holarctica* strain LVS (LVS) and *F.*

95 *tularensis* subsp. *novicida* strain U112 (novicida) were obtained from the CDC. *F. tularensis*

96 subsp. *tularensis* strain Schu S4 (Schu S4) was obtained from the Rocky Mountain Laboratories,

97 MT. *F. tularensis* subsp. *tularensis* clinical strains 1 through 10 were obtained from the

98 Departments of Public Health in Utah and New Mexico (Table 1.). All *F. tularensis* strains used

99 in this study were grown on modified Mueller Hinton agar (Difco) supplemented with 0.025%

100 ferric pyrophosphate (Sigma), 0.02% IsoVitaleX (Becton Dickinson), 0.1% glucose and 0.025%

101 calf serum (Invitrogen-Gibco) at 37°C with 5% CO2, or modified Mueller-Hinton broth

102 (supplemented as described above) with aeration at 37°C.

103 **Cell lines and culture conditions.** *A. castellanii* (ATCC 30234) were grown axenically in PYG

104 broth (12) to 90% confluency at 23°C in the dark in 75 cm^2 tissue culture flasks (Falcon). The

105 amoebae were harvested before use by rapping the flask sharply to bring them into suspension

106 and the number of viable cells was determined as described previously (12). To induce Amoeba

107 encystment, *A. castellanii* were suspended in High Salt Buffer (HS) for 3 days as described

108 previously (4, 33).

109 **Entry and adherence assays.** *A. castellanii* entry and adherence assays were carried out as

110 described previously (10, 13) in 24-well tissue culture plates (Costar). Briefly, *A. castellanii*

111 were seeded into plates at a concentration of 2 X 10^5 cells per well and allowed to adhere

112 overnight at 23°C. The Amoebae were washed with HS buffer (4) and incubated in 1 ml of HS

113 buffer for 1 hr at 37°C with 5% CO_2 prior to infection. *F. tularensis* overnight cultures were

114 added to amoeba trophozoites at a multiplicity of infection (MOI) of 10. After co-incubation for

115 30 min, the cells were washed once and incubated in HS buffer plus 100µg gentamicin per ml for

116   2 hr at 37°C and 5% CO_2. The amoebae were then washed once to remove gentamicin and lysed

117   by incubation in 1% saponin for 5 min (Sigma) followed by vigorous pipetting. Dilutions were

118   plated on supplemented Mueller Hinton agar to determine viable colony forming unit (CFU)

119   counts. Adherence assays were carried out in a similar manner except that after the amoeba were

120   infected with the bacteria for 30 minutes, they were immediately washed 3 times with HS buffer

121   prior to lysis. Percent entry was calculated as follows: ($CFU_{intracellular}$/ $CFU_{Inoculum}$) X 100.

122   Saponin (1%) had no effect on the viability of *F. tularensis* strains and all strains used displayed

123   comparable levels of killing by gentamicin.

124   **Intracellular survival assays.** Intracellular survival assays were carried out in a manner similar

125   to that of the entry assays with the following modifications: after gentamicin treatment, fresh HS

126   buffer was added and the amoebae were incubated at 37°C with 5% CO_2, lysed and plated at the

127   indicated times post infection. Survival is expressed as the percentage of CFU present

128   intracellularly at each time point (T_x) compared to time zero (2.5 hr), i.e. percent survival= (CFU

129   T_x/ CFU T_0) X 100.

130   **Long-term survival assays.** Amoebae were infected with *F. tularensis* strains in 6-well plates

131   (Costar) as described above. After gentamicin treatment and washing, 3 ml of fresh HS buffer

132   were added to each well and the plates incubated at 37°C with 5% CO_2 for up to 3 weeks. To

133   recover bacteria, plates were spun down for 5 min at 100 X *g* and the HS buffer in each well was

134   replaced with 3 ml PYG medium supplemented with IsoVitaleX. The plates were re-incubated at

135   37°C with 5% CO_2 until the wells became turbid or for 72 hr (whichever occurred first). The

136   cultures were then plated on Mueller Hinton agar and samples were gram-stained to confirm

137   presence of *F. tularensis*. To inhibit amoeba encystment, *A. castellanii* trophozoites were treated

138   with 25µg/ml cycloheximide concurrently with gentamicin treatment for 2 hr then washed before

139   addition of HS buffer. To inhibit amoeba excystment, *A. castellanii* cysts were treated with

140   25μg/ml cycloheximide in HS buffer for 1 hr prior to addition of PYG medium for bacterial

141   recovery.

142   **Cytotoxicity assays.** *A. castellanii* were seeded in 96-well plates at a concentration of 5 X 10^4

143   cells per well and allowed to adhere overnight at 23^oC. The medium was replaced with HS buffer

144   and the amoebae were incubated at 37^oC with 5% CO_2 for 1 hour prior to infection. *F. tularensis*

145   overnight cultures were added to cells at MOIs of 10. After co-incubation for 1 hr at 37^oC and

146   5% CO_2, the medium was replaced with fresh HS buffer. Cell death was quantified

147   colorimetrically using the Cyto-Tox96 lactate dehydrogenase (LDH) release kit (Promega)

148   according to the manufacturer's recommendations.

149   **Fractionation and HPLC analyses.** Amoebae-*Francisella* co-cultures were collected at 6 hr

150   post infection, size-fractionated and concentrated using Amplicon filters (Millipore) with 100

151   kD, 30 kD and 3 kD pore sizes sequentially. Twenty-five ml aliquots of *F. tularensis- A.*

152   *castellanii* co-culture media were loaded into the largest pore sized filter and centrifuged. The

153   eluate was then loaded into the smaller pore sized filter and the sequence was repeated until the

154   last eluate was filtered through the smallest pore size filter. Centrifugation was done at 740 X *g*

155   for 5-60 min according to the manufacturer's instructions. Total protein concentrations were

156   determined separately for each eluted fraction. 150 and 250 μg of total protein from each fraction

157   were then diluted with running buffer and loaded separately on SuperDex 200 10/300 high-

158   pressure liquid chromatography (HPLC) columns (GE Healthcare) and sub-fractioned according

159   to the manufacturer's recommendations. The sub-fractions were either concentrated or diluted

160   with PBS to 1 ml final volumes.

161   **Protein Identification.** The proteins present in *F. tularensis-Amoeba* co-culture sub-fractions

162   were identified commercially by ProtTech, Inc. (Norristown, PA) using Nano LC-MS/MS

163   peptide sequencing technology. The Proteins were first denatured by addition of 8M urea

164   followed by reduction of the Cys residues in the solution with 20mM dithiothreitol (DTT) and

165   alkylated with 20mM iodoacetamide. The samples were then diluted to 2M urea with 100mM

166   ammonium bicarbonate (pH 8.5) and the proteins digested by addition of sequencing-grade

167   modified trypsin (Promega, Madison, WI). The resulting peptide mixture was desalted with a

168   PepClean spin column ((Pierce, Rockford, IL), and analyzed using a LC-MS/MS system, in

169   which an HPLC reverse phase C18 column with a 75 micrometer inner diameter was on-line

170   coupled with an ion trap mass spectrometer (Thermo, Palo Alto, CA). The mass spectrometric

171   data acquired from LC-MS/MS analyses were used to search against the recent Non-Redundant

172   Protein Database from GenBank (http://www.ncbi.nlm.nih.gov/) using ProtTech's ProtQuest

173   software package. Except where specified, all other chemicals used were purchased from Sigma

174   (St. Louis, MO).

175   **Microscopy.** To evaluate amoebae encystment, *A. castellanii* were infected with overnight

176   cultures of *F. tularensis* strains at an MOI of 20 in triplicate. The infection was allowed to

177   proceed for 2 hours and the infected amoebae were examined using a Nikon TE300 light

178   microscope with differential interference contrast options, attached to a digital screen. The

179   morphology of the infected amoebae were noted and quantified by counting the number of

180   amoebae trophozoites and cysts present in three random fields per infected well. Transmission

181   electron microscopy (TEM) was used to examine the ultrastructure of *A. castellanii* infected with

182   *F. tularensis* strains. For TEM, *A. castellanii* were infected at an MOI of 10 for 10 min at $37^{o}C$

183   with 5% CO_2, washed twice with HS buffer and incubated in fresh HS buffer at $37^{o}C$ and 5%

184   CO_2. After incubation for the indicated durations, the amoebae were suspended in medium with a

8

185   rubber policeman, pelleted by centrifugation for 2 minutes at 740 X g at 25°C, fixed and

186   prepared for electron microscopy as previously described (12, 19). To track endosomal

187   vacuoles, *A. castellanii* trophozoites were pre-loaded with 10nm SPI-Mark colloidal gold

188   unconjugated particles (SPI Supplies) overnight before infection. The samples were suspended in

189   2% glutaraldehyde for 1 hr, treated with 1% OsO_4 for 2 hr and then postfixed with 0.5% uranyl

190   acetate at 4°C overnight. The cells were embedded and sectioned as described previously (19).

191   Immunofluorescent microscopy (IF) was performed as described previously (18). Briefly, *A.*

192   *castellanii* lysosomes were preloaded with LysoTracker red DND-99 (Invitrogen-Molecular

193   Probes) for 30 min before infection, infected for different durations then washed with HS buffer

194   and fixed with 4% paraformaldehyde in 100mM phosphate buffer (pH 7.4). The amoebae were

195   permeabilized with 95% ethanol, blocked with 3% BSA, and stained with a chicken anti–*F.*

196   *tularensis* polyclonal antibody. To stain infected mature amoebae cysts, the cysts were washed

197   with high salt buffer and centrifuged onto slides using a Cytospin centrifuge (Thermo Fisher) for

198   3 min at 1500 X g, fixed for 30 min with 4% paraformaldehyde, then permeabilized for 30-60

199   min with 95% ethanol. The amoebae were visualized by fluorescent microscopy using a Zeiss

200   AxioVert microscope after staining with Alexa Fluor–coupled secondary antibodies (Invitrogen-

201   Molecular Probes). Z-stacks of slices (1-µm thick) were captured using Zeiss Axiovision

202   software and reconstructed 3D images were assembled by use of Volocity software (version 5.1;

203   Perkin Elmer- Improvision).

204   **Statistical analyses.** Mean and standard deviation were calculated from triplicate samples for

205   representative experiments. All experiments were repeated at least three times, unless otherwise

206   noted. Significance was determined by analysis of variance using the paired Student *t* test. *P*

207   values of <0.05 were considered significant.

208   **RESULTS**

209   ***F. tularensis* strains enter and replicate in *A. castellanii* with different efficiencies.** To

210   determine if pathogenic *F. tularensis* strains enter and survive in *A. castellanii*, we infected

211   amoebae with 10 clinical isolates of *F. tularensis* subsp. *tularensis* strains chosen from

212   geographically and temporally separate tularemia outbreaks (Ft 1-10, Table 1) and the laboratory

213   strain *F. tularensis* subsp. *tularensis* Schu S4 (Schu S4). For comparison purposes, we also

214   infected *A. castellanii* with the commonly used laboratory strains *F. tularensis* subsp. *novicida*

215   (novicida) and *F. tularensis* subsp. *holarctica* strain LVS. Our results demonstrate that *F.*

216   *tularensis* strains associate with, enter and survive in *A. castellanii* with varying efficiencies (Fig.

217   1). LVS was significantly less efficient at adherence, entry and survival in *A. castellanii*

218   compared with the other strains tested ($P \leq 0.02$). We therefore chose to represent infection

219   experiments relative to LVS. *F. tularensis* strains varied in their ability to adhere to *A.*

220   *castellanii* with the least efficient strains, Ft-4 and Ft-9, adhering at a rate 1.5 - 2 times higher

221   than LVS ($P = 0.05$) and the most efficient strains, Ft-1 and Ft-2, adhering at a rate 7 - 10 times

222   higher than LVS ($P = 0.02$) (Fig. 1A). With the exception of strains Ft-4 and Ft-7 which entered

223   at rates comparable to LVS, all the strains tested consistently entered *A. castellanii* at a rate 5 -

224   50 times higher than LVS ($P \leq 0.05$) (Fig. 1B). We also examined the ability of *F. tularensis*

225   strains to survive and replicate in *A. castellanii* at 24 hours post infection (Fig. 1C). For LVS

226   only half of the CFU at time zero (2.5 hr) were recovered after 24 hours. This data is

227   significantly different from the data described by Abd et al. (1) and can be attributed to their use

228   of a rich medium which we observed allowed for replication of LVS extracellularly (Fig. 2A).

229   Strains Ft-4 and Ft-6 were recovered at a similar rate as LVS, whereas the rest of the strains

230   varied widely in their ability to survive and replicate. Ft-3, Ft-9 and Ft-2 were recovered at a rate

231   1.2 – 1.5 times higher than LVS after 24 hr ($P = 0.05$). Ft-5, Ft-8, Ft-10, Schu S4 and novicida

232   were recovered at a rate 2 - 5 times higher ($P = 0.02$) and Ft-1 and Ft-7 were recovered at a rate 7

233   - 8 times higher than LVS after 24 hr ($P = 0.01$). With the exception of Ft-7, all other strains

234   tested correlated in their ability to attach, enter and survive in *A. castellanii*. Though Ft-7 showed

235   a relatively high rate of immediate attachment to the amoebae, the CFU recovered at time zero

236   were consistently low. Interestingly, by 24 hr post infection, Ft-7 CFU were approximately 7

237   times higher than the CFU recovered at time zero. These data suggest that either Ft-7 enters at a

238   low rate but replicates very efficiently or that the strain is killed early in the infection but is able

239   to recover and replicate after a lag phase. We were unable to assess intracellular growth past 24

240   hr by CFU counts since the majority of the amoebae were encysted by then and were extremely

241   resistant to lysis, both chemical and mechanical, consistent with previous reports (28-30).

242   **Pathogenic *F. tularensis* strains are present in spacious vacuoles at 30 min post-infection.**

243   To characterize the ultrastructure of pathogenic *F. tularensis* vacuoles in amoeba, we infected *A.*

244   *castellanii* trophozoites with LVS, novicida, Schu S4 and Ft-1 and examined them by TEM. By

245   30 min post infection the majority of vacuoles containing novicida, Schu S4 and Ft-1 were

246   significantly more spacious ($P \leq 0.02$) (Fig. 3A and Table 2) than vacuoles containing LVS. To

247   determine if *F. tularensis* strains disrupt the endosomal pathway, we preloaded amoeba

248   lysosomes with nanogold particles prior to bacterial infection, which allowed us to assess the

249   frequency of lysosomal fusion (Fig. 3B). We were able to confirm a statistically significant

250   correlation between tight vacuole formation and the presence of nanogold particles and we found

251   that by 2 hours post infection, 82% of bacterial tight vacuoles formed by all four strains co-

252   localized with nanogold particles ($P = 0.02$) (Table 2). This number rose to 88% at 24 hours post

253   infection ($P = 0.01$), suggesting that tight vacuoles are lysosomal in nature while spacious ones

11

254   are not. At 24 hours post infection, over 90% of amoeba infected with novicida, Schu S4 and Ft-

255   1 contained bacteria present in spacious vacuoles compared to 57% of amoeba infected with

256   LVS (Table 2). By this stage the majority of *A. castellanii* infected with novicida, Schu S4 and

257   Ft-1 were encysted and intact bacteria were observed within the double wall layers of the

258   amoebae cysts. An "early" or "young" cyst containing bacteria is shown in Fig. 3C.

259   Trophozoites still present at this point contained bacteria in spacious vacuoles, and multiple

260   vacuoles containing bacteria were observed within the same trophozoite (Fig. 3D). The bacterial

261   structure appears typical to that described for other pleiomorphic bacteria. Similar to what has

262   been previously described with LVS, novicida, Schu S4 and Ft-1 recruit mitochondria and

263   membrane structures suggestive of the endoplasmic reticulum (ER) to the bacterial vacuoles

264   (Fig. 3E), which display intact phoagosomal membranes (Fig. 3F).

265   **LVS co-localizes with the lysosomal marker LysoTracker Red.** To confirm our observations

266   that LVS containing tight vacuoles are lysosomal in nature, we pre-loaded amoeba lysosomes

267   with the lysosomal marker LysoTracker red and infected them with LVS and novicida strains

268   expressing GFP. After 30 min post infection, 26% of novicida colocalized with LysoTracker red

269   compared to 48% of LVS ($P = 0.02$) (Fig. 4A and Table 2). By 2 hr post infection, 43% of

270   novicida colocalized with LysoTracker red compared with 89% of LVS ($P = 0.01$) (Table 2).

271   These data confirm that LVS reside predominantly in lysosomal vacuoles while novicida appears

272   able to block lysosomal fusion in *A. castellanii.*

273   **Novicida is present in *A. castellanii* cysts after 7 days post-infection.** To confirm that the

274   structures observed by TEM within the double walls of amoeba cysts are bacteria, we infected *A.*

275   *castellanii* with either LVS and novicida or LVS and novicida strains expressing GFP, and

276   incubated them for various times post infection. At the indicated time points, amoeba cysts were

12

277   either fixed and visualized directly (GFP expressing strains) or stained with a chicken anti-*F.*

278   *tularensis* polyclonal antibody prior to processing for IF and visualization. *A. castellanii* cysts

279   stained positive for novicida but not LVS after 24 hr and 7 days post infection (Fig.5). *A.*

280   *castellanii* cysts infected with GFP-expressing novicida were only positive for novicida after 24

281   hours (data not shown). Since GFP is not expressed at 7 days post-infection, the novicida present

282   in *A. castellanii* cysts may either have lost the plasmid or may be metabolically inactive.

283   **Recovery of viable *F. tularensis* strains from *A. castellanii* cysts 21 days post-infection.**

284   Demonstrating the presence of viable *F. tularensis* in *A. castellanii* cysts by intracellular growth

285   assays was not possible due to increased resistance of amoeba cysts to lysis as previously

286   reported (28-30). To circumvent this problem, we designed an experiment based on our

287   observations that all the *F. tularensis* strains we tested replicate vigorously in PYG medium (Fig.

288   2A) but not in HS buffer (data not shown). This allowed us to determine whether virulent *F.*

289   *tularensis* strains were able to survive longer than 24 hours in *A. castellanii*. *A. castellanii*

290   trophozoites were infected with novicida, LVS, Schu S4, Ft-1 and Ft-7. After gentamicin

291   treatment, fresh HS buffer was added to each well and the amoebae were allowed to incubate for

292   varying durations. Weekly, infected cysts were spun down and the buffer replaced with PYG.

293   Presence of the rich PYG medium enabled the amoebae to excyst and the bacteria present in the

294   cysts to be released into the medium where they could replicate. As shown in Table 3, viable

295   novicida, Ft-1 and Ft-7 were recovered up to three weeks post infection. We observed about 80%

296   of the amoeba excysted after 24 hr of the addition PYG broth, consistent with previous data

297   describing the kinetics of *A. castellanii* excystment (28). We did not observe bacterial turbidity

298   in the infected amoebae wells, nor were we able to recover bacterial CFUs before 36-48 hr after

299   replacement of HS buffer with PYG. This data suggests that *F. tularensis* were present

300   intracellularly and not just associated with the outer surface of the cysts. To confirm these

301   results, we blocked infected amoeba excystment in *F. tularensis*-infected cysts using

302   cycloheximide as previously described (9), and did not recover any bacteria upon addition of

303   PYG (Table 3). Not unexpectedly, we were only able to recover viable LVS up to 3 days post

304   infection, consistent with our CFU assays demonstrating that the LVS strain is attenuated in

305   amoeba infections.

306   ***F. t. novicida* and virulent *F. tularensis* strains induce the rapid encystment of *A. castellanii*.**

307   In contrast to infection with LVS (Fig. 6B), our initial observations of *A. castellanii* infected with

308   novicida and Schu S4 showed that a large number of amoebae trophozoites began encysting

309   within 2 hours of infection (Fig. 6C and E). Although *A. castellanii* is known to encyst in

310   response to starvation, desiccation and other adverse environmental conditions (24) and can be

311   induced to artificially encyst after ~3 days in the laboratory artificially by growth in HS buffer

312   (4) (also known as "encystment buffer") (33), the presence of novicida and Schu S4 rapidly

313   accelerated this natural phenomenon. The Rapid Encystment Phenotype (REP) we observed also

314   occurred in response to *F. tularensis* strains even when the amoebae were grown xenically in

315   presence of heat killed *E. coli* as a food source as previously described (48). In addition, REP

316   was not associated with an increase in cytotoxicity, confirming that the amoebae were encysted

317   and not dead (data not shown). We investigated the ability of *F. tularensis* clinical isolates (Ft 1-

318   10) to cause the rapid encystment of *A. castellanii* trophozoites compared with novicida, LVS

319   and Schu S4. *F. tularensis* clinical isolates varied in their ability to cause REP (Fig. 6). Further,

320   Ft-3 (Fig. 6D), Ft-4 and Ft-9 did not cause REP while Schu (Fig. 6E), Ft-2 (Fig. 6F), Ft-8 (Fig.

321   6G) and Ft-10 (Fig. 6H) caused the highest REP levels. Interestingly, out of the 10 clinical

322   strains tested, 5 strains (50%) were able to induce a significantly higher level of *A. castellanii*

14

323 encystment compared with uninfected trophozoites or trophozoites infected with LVS ($P \leq 0.03$)

324 (Fig. 7). Novicida however, caused the highest levels of REP observed, a phenomenon that may

325 be explained by the rapid growth of novicida compared to type A *F. tularensis* strains. It is

326 interesting to note that the same 5 strains that induced higher levels of encystment (Ft-1, Ft-2, Ft-

327 7, Ft-8 and Ft-10), were associated with higher rates of attachment, entry and survival in *A.*

328 *castellanii* than all other strains tested.

329 **A protein fraction isolated from *F. tularensis*- *A. castellanii* co-cultures is responsible for**

330 **the rapid encystment of *A. castellanii*.** To investigate whether the rapid amoebae encystment

331 we observed was a result of direct contact between the bacterium and amoeba or mediated by

332 soluble factor(s), we used a transwell culture system to physically separate the amoebae

333 monolayer from the bacteria by using an insert with a 0.2µm filter. The inability of *F. tularensis*

334 strains to pass through the filter was confirmed by plating for viable CFUs (data not shown). In

335 the absence of cell-cell contact using the transwell system, *A. castellanii* trophozoites still

336 induced REP in response to the same bacterial strains (data not shown). These data suggest that

337 REP may be caused by factor(s) secreted by *F. tularensis* strains in response to *A. castellanii*

338 and/or factor(s) secreted by *A. castellanii* in response to bacterial infection. Since spent bacterial

339 culture media did not confer REP (data not shown), we concluded that cross-talk occurring

340 between *F. tularensis* and *A. castellanii* is necessary for induction of the encystment phenotype.

341 To determine whether the soluble factors in REP were proteinaceous in nature, bacteria-amoeba

342 co-cultures were boiled or subjected to a 30 min treatment with proteinase K. Both of these

343 treatments resulted in abrogation of REP (data not shown) suggesting the factor(s) responsible

344 are proteins. The co-culture media from *A. castellanii* infected with LVS, novicida, Schu S4, Ft-

345 1, Ft-2, Ft-7 and Ft-8 were then fractionated and analyzed by HPLC. Four different size fractions

346 ranging from ≥100kD, <100kD ≥30kD, <30kD ≥3kD to <3kD were obtained for each strain.

347 Addition of these 4 fractions separately to naïve *A. castellanii* trophozoites demonstrated that the

348 ≥3kD (<30kD) fractions from novicida, Schu S4, Ft-1, Ft-7 and Ft-8 infections induce REP (Fig.

349 8). The <100kD (>30kD) fraction from novicida was also able to confer REP suggesting there

350 may be slight size differences between the factor(s) produced by type A strains and novicida.

351 Addition of the ≥3kD (and <30kD) fraction from the LVS- *A. castellanii* co-culture did not result

352 in REP and resembled cultures treated with other fractions or medium alone.

353 **REP fractions from novicida and Schu S4 contain proteins that mediate** *A. castellanii*

354 **encystment.** To identify the proteins that may be responsible for the observed REP, we had the

355 REP fractions from novicida and Schu S4 analyzed commercially by LC-MS/MS. In parallel, we

356 also analyzed fractions of the same size isolated from uninfected *A. catellanii* trophozoites, *A.*

357 *castellanii* cysts and laboratory-grown novicida. Interestingly, we identified the same proteins in

358 REP fractions from novicida and Schu S4. LC-MS/MS analyses revealed 4 unique *A. castellanii*

359 proteins that were only present in the *A. castellanii-F. tularensis* REP fraction and one protein

360 (subtilisin-like serine proteinase) that was present in both the REP fraction and the *A. castellanii*

361 cyst fraction (Table 5). We were able to infer that the subtilisin serine proteinase is present in the

362 REP fractions at four times the amount it was present in the *A. castellanii* cyst fraction because

363 the number of peptides sequenced by LC-MS/MS from each protein can be used as an indication

364 for relative abundance of proteins in a sample.   This serine proteinase shows 97% identity and

365 98% homology at the C-terminal region to a previously identified subtilisin family serine

366 proteinase that has recently been shown to mediate the encystation of *A. castellanii* (33, 34).

367 Interestingly, the REP fraction also contained Ubiquitin and an Ubiquitin fusion protein. The

368 proteasome/ Ubiquitin system has been shown be involved in the encystment of amoeba, a

369   process requiring extensive protein degradation (22). The other 2 proteins identified in the

370   fractions were Actin and the α-chain of Profilin II. Profilin is an actin-binding protein involved

371   in the dynamic turnover and restructuring of the actin cytoskeleton (27, 51). The protein is

372   normally found in association with monomeric actin in *Acanathamoeba* (27). We also identified

373   a large protein, which is predicted to be a chaperone belonging to the DnaK/HSP 70 family.

374   Considering that the size of this protein is larger that 30 kD, it is unlikely that it is actually

375   secreted, most probably it was released into the medium as a result of amoeba lysis during

376   encystment. In addition to *A. castellanii* proteins, we also identified 7 *F. tularensis* proteins that

377   were only present in the REP fraction and not in laboratory grown *F. tularensis.* Since most of

378   these genes are annotated in the databases as "hypothetical proteins", we are not able to speculate

379   on their function. We are currently constructing mutations in the genes encoding these proteins

380   and expressing them in vitro to identify their role in inducing amoeba encystment.

381

382

383

384

385

386

387

388

389

390

391

DISCUSSION

392

393   *Francisella tularensis* was first identified as the cause of a plague-like outbreak in ground

394   squirrels in 1911(32). There has been a rising interest in *F. tularensis* (49) in recent years , due in

395   a large part to the recognition of *F. tularensis* as a potential bioterrorism agent (47). *F.*

396   *tularensis* exhibits an extremely broad host range, and the bacterium is known to infect hundreds

397   of wildlife species (41), which facilitates human infections. However, the acute nature of the

398   infections in vertebrate and invertebrate hosts identified so far suggests that the reservoir(s) of *F.*

399   *tularensis* in the environment have not been identified (36). Previous studies have correlated

400   tularemia outbreaks with aquatic environments (5, 6) and suggested that environmental amoebae

401   may serve as bacterial reservoirs in nature (40). So far, only two such studies examined the

402   survival of *F. tularensis* strains in the amoeba *Acanthamoeba castellanii* (1, 43). Though these

403   studies concluded that *F. tularensis* LVS and novicida are able to survive in *A. castellanii* for

404   weeks post-infection, the authors used a rich medium that allowed for the replication of the

405   bacteria extracellularly for all survival experiments, making it impossible to discern true long-

406   term intracellular survival. In addition, the authors did not characterize the interaction of virulent

407   *F. tularensis* strains with amoeba, leaving unanswered the question of whether amoebae can

408   serve as environmental reservoirs for *F. tularensis* strains pathogenic for humans.

409

410   In the present study, we conducted a detailed characterization of the interaction of

411   multiple *F. tularensis* strains with the amoeba *A. castellanii*, and have demonstrated for the first

412   time, the ability of fully virulent strains to enter and survive in amoeba. To ensure that we were

413   able to quantify long-term survival without confounding factors, all our experiments were

414   performed in a high salt buffer that supports the survival of *A. castellanii* but does not allow for

18

415   the growth of *F. tularensis* (data not shown). In addition to the most commonly used laboratory

416   strains, LVS, Schu S4 and novicida, the interaction of 10 clinical strains, isolated from human,

417   rodent and lagomorph outbreaks in New Mexico and Utah were also examined. To maximize our

418   chances of obtaining disparate isolates, we chose strains that were isolated from geographically

419   separate outbreaks over a 10-year period. Since their isolation, these strains have not been

420   manipulated in the laboratory, and have remained largely uncharacterized. Our results

421   demonstrate, for the first time that fully virulent *F. tularensis* strains can associate with and enter

422   *A. castellanii,* albeit with disparate efficiencies. Further, we have demonstrated that long-term

423   survival of pathogenic *F. tularensis* isolates in amoeba is dependent on induction of amoeba

424   encystment. Not surprisingly, we observed that LVS is the least efficient at both association and

425   entry, consistent with the non-pathogenic nature of this isolate. However, *F. tularensis* strains in

426   general replicated much less in *A. castellanii* than other amoeba-resistant bacteria such as *M.*

427   *avium* and *L. pneumophila* (10, 11). The variation in the ability of clinical *F. tularensis* strains to

428   associate with and survive in *A. castellanii* suggests that more than one environmental host may

429   exist for *F. tularensis*

430

431   To assess downstream events after bacterial entry, we examined the ultrastructure of *F.*

432   *tularensis* infected amoeba by TEM. We observed that *F. tularensis* strains were associated with

433   two types of bacterial vacuoles, tight vacuoles, which directly conform to the bacterial shape,

434   and spacious vacuoles, usually associated with multiple bacteria. Quantification of the type of

435   bacterial vacuoles associated with each strain revealed that LVS was enclosed in tight vacuoles

436   at a rate four times higher than novicida, Schu and Ft-1. In addition, though we were unable to

437   calculate the frequency of lysosomal fusion with individual strains due to the low numbers of

438   trophozoites present after 24 hours of infection, we found that 88% of tight vacuoles colocalized

439   with nanogold particles. These data suggest that spacious vacuoles may not be lysosomal in

440   nature and that enclosure within these vacuoles may provide a survival advantage to novicida,

441   Schu and Ft-1. This would be similar to what has been described for survival of *Salmonella* in

442   macrophages (26). We were able to calculate the frequency of lysosomal fusion using IF and

443   found that by 2 hr post infection 89% of LVS vacuoles colocalize with lysosomes compared to

444   43% of novicida vacuoles, consistent with our TEM observations.

445

446   Even though the majority of *A. castellanii* trophozoites encyst within 24 hours after

447   infection, we have demonstrated for the first time the ability of virulent *F. tularensis* strains to

448   survive in the amoebal cysts for up to three weeks post infection. Surprisingly, viable Schu S4

449   was only recovered up to two weeks post infection. This observation may be explained by the

450   fact that the Schu S4 strain has been propagated under laboratory conditions for almost 70 years

451   since its initial isolation from a clinical case (8, 16), and some loss of virulence is to be expected.

452   This extended propagation may account for the inability of Schu S4 to survive past two weeks

453   compared with the other type A strains (e.g. Ft-1 and Ft-7) that have been only minimally

454   manipulated in the laboratory. Unlike *Mycobacterium* and *Legionella* spp., the *F. tularensis*

455   strains examined do not replicate to a high degree in *A. castellanii* cysts but appear to survive by

456   inducing amoeba encystment. It is still possible that these *F. tularensis* strains may indeed grow

457   to large numbers in amoebae trophozoites when abundant nutrients are present, and in fact many

458   amoeba-resistant microorganism are known to be endosymbiotic or lytic in a given amoeba

459   depending on environmental conditions (23, 24). Another plausible hypothesis is that amoeba-

460   resistant microorganisms have developed multiple approaches to environmental survival. Our

461   data using the eukaryotic protein synthesis inhibitor, cycloheximide, suggest that the ability to

462   cause amoeba encystment is necessary for the survival of the *F. tularensis* strains tested in *A.*

463   *castellanii*. Alternatively, cycloheximide may be acting by preventing the synthesis of an

464   amoebal protein or by blocking a protein-mediated process the bacteria needs to survive upon

465   internalization. Considering the drought resistance and hardiness of amoeba cysts, cyst formation

466   could enable intracellular *F. tularensis* to survive desiccation and food shortage in the

467   environment. *F. tularensis* could then be transmitted orally to animals that drink from water

468   contaminated with amoeba cysts. Infected animals would then complete the environmental cycle

469   by fecal shedding of *Francisella* in or near aquatic environments, which are prime amoeba

470   habitats (2, 3). Though amoeba encystment in response to bacterial infection has been reported

471   previously, this usually occurs in presence of a high bacteria to amoeba ratio (52). To explore the

472   cause of the rapid encystment of *A. castellanii* trophozoites in response to *F. tularensis* infection,

473   we verified that the amoebae were actually encysted and not dead by conducting cytotoxicity

474   assays comparing infected and uninfected *A. castellanii* (data not shown). We also confirmed

475   that the amoebae were not simply encysting in response to overwhelming numbers of bacteria or

476   lack of nutrients, by reducing the MOI of infection and/ or growing the amoeba xenically in

477   presence of heat killed *E. coli* as a food source (48). At MOIs of 1 and 5 we were not able to

478   recover viable organisms following *F. tularensis* infections but we still obtained high levels of

479   amoeba encystment. Interestingly, it has been observed that *A. castellanii* does not usually

480   undergo encystment in response to low levels of bacterial replication (14, 54) (Ling Yan,

481   personal communication). This data suggests that the encystment was specific to *F. tularensis*

482   and not simply due to the presence of high numbers of bacteria or low nutrient levels. In

483   addition, using a transwell culture system, we demonstrated that encystment does not require

484   direct bacteria-cell contact, as REP still occurred even though the bacteria were physically

485   separated from the amoeba trophozoites.

486

487   Quantification of encystment showed that *F. tularensis* strains varied in their ability to

488   cause REP. Novicida showed the highest level of REP, most likely because of its rapid rate of

489   growth compared to LVS and virulent *F. tularensis* strains. Along with novicida, Schu S4, Ft-1,

490   Ft-2, Ft-7, Ft-8 and Ft-10 caused very high rates of REP, while LVS, Ft-3, Ft-4 and Ft-6 did not.

491   Since all virulent *F. tularensis* strains and LVS showed similar rates of growth, the failure of

492   LVS, Ft-3, Ft-4 and Ft-6 to induce rapid encystment in amoeba cannot be attributed to the

493   number of CFU present. It is interesting to note that *F. tularensis* strains causing the highest

494   levels of encystment were also the strains showing the highest levels of attachment, entry and

495   replication in *A. castellanii*.

496

497   Induction of REP appears to involve proteins produced as a result of *F. tularensis-A.*

498   *castellanii* cross-talk. Our experiments show that addition of co-culture media from *A. castellanii*

499   and *F. tularensis* strains that cause REP confer REP on naïve amoeba trophozoites while spent

500   culture media from the same *F. tularensis* strains alone does not. These data also suggest that

501   soluble factor(s) secreted into the co-culture medium by the bacteria and/or the amoeba mediate

502   the phenotype. Consequently, we conducted some preliminary analyses of the co-culture media

503   in order to narrow down the size and nature of the fraction responsible for inducing the

504   phenotype. We found factors responsible for inducing encystment to be between 3-30 kD and

505   concluded that the active component(s) of the fraction that induce REP are likely to be protein(s)

506   as boiling or proteinase K treatment abrogated the activity of the fraction as evidenced by the

507   loss of REP. LC-MS/MS analyses of the REP-inducing fraction revealed that the same proteins

508   are present in REP-inducing fractions from novicida and Ft-1. We identified a 16.5 kD subtilisin-

509   like serine proteinase that was present in REP fractions at 4 times the quantity it was present in

510   media from *A. castellanii* cysts. This subtilisin-like serine proteinase shows 97% identity and

511   98% homology at the C-terminus containing the peptidase S8 region (pfam0082) to a previously

512   identified 33 kD Encystment-Mediating Serine Proteinase (EMSP) that has recently been shown

513   to mediate the encystation of *A. castellanii* (33, 34). This suggests that our protein may be a

514   cleavage product of EMSP. Subtilases have been associated with autophagosomes (39, 50) and

515   EMSP si-RNA-treated *A. castellanii* show defects in substance degradation and autophagy

516   maturation during encystation (34). The abundance of EMSP-like protein in the REP fraction

517   suggests autophagy may be activated in *A. castellanii* in response to *F. tularensis* infection. The

518   REP fraction also contained Ubiquitin and a Ubiquitin fusion protein. The proteasome/ Ubiquitin

519   system is responsible for degradation of proteins (22, 38). It is likely that this system is used to

520   degrade proteins present in trophozoites that will not be required in the emerging cysts. The other

521   2 proteins identified in the fractions were Actin and the α-chain of Profilin II. Profilin is an

522   actin-binding protein involved in the dynamic turnover and restructuring of the actin

523   cytoskeleton (27, 51). This protein is normally found in association with monomeric actin in

524   *Acanathamoeba* (27). The presence of both of these proteins in the REP fraction is likely due to

525   the cytoskeletal rearrangements that occur upon encystment and not due to their secretion.

526

527   In addition to *A. castellanii* proteins, we also identified seven *F. tularensis* proteins that

528   were only present in the REP-inducing fraction and not in the media of laboratory grown *F.*

529   *tularensis.* We are currently characterizing these proteins to identify the role they play in

530   induction of amoeba encystment. Previous studies have shown that interactions of a number of

531   intracellular bacterial pathogens with amoebae result in an enhancement in the pathogen's ability

532   to enter and survive in mammalian cells (7, 11, 12). Since it is likely that bacteria-amoebae cross

533   talk results in amoeba encystment, this process could also result in the up-regulation of bacterial

534   virulence factors that may be required for subsequent entry and survival in mammalian cells in

535   addition to enhancing the ability of *F. tularensis* to persist in the environment.

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550

551

552

566

567

568

569

570

571

572

573

574

575
576

REFERENCES

577

578   1.   **Abd, H., T. Johansson, I. Golovliov, G. Sandstrom, and M. Forsman.** 2003. Survival
579   and growth of *Francisella tularensis* in *Acanthamoeba castellanii.* Appl. Environ.
580   Microbiol. **69:**600-606.
581   2.   **Anderson, O. R.** 2002. Laboratory and field-based studies of abundances, small-scale
582   patchiness, and diversity of gymnamoebae in soils of varying porosity and organic
583   content: evidence of microbiocoenoses. J. Eukaryot. Microbiol. **49:**17-23.
584   3.   **Arias Fernandez, M. C., E. Paniagua Crespo, M. Marti Mallen, M. P. Penas Ares,**
585   **and M. L. Casro Casas.** 1989. Marine amoebae from waters of northwest Spain, with
586   comments on a potentially pathogenic euryhaline species. J. Protozool. **36:**239-241.
587   4.   **Band, R. N. a. S. M.** 1969. The respiratory metabolism of *Acanthamoeba*
588   *rhysodes* during encystation. J. gen. Microbiol. **59:**351-358.
589   5.   **Berdal, B. P., R. Mehl, H. Haaheim, M. Loksa, R. Grunow, J. Burans, C. Morgan,**
590   **and H. Meyer.** 2000. Field detection of *Francisella tularensis.* Scand. J. Infect. Dis.
591   **32:**287-291.
592   6.   **Berdal, B. P., R. Mehl, N. K. Meidell, A. M. Lorentzen-Styr, and O. Scheel.** 1996.
593   Field investigations of tularemia in Norway. FEMS Immunol. Med. Microbiol. **13:**191-
594   195.
595   7.   **Brieland, J., M. McClain, L. Heath, C. Chrisp, G. Huffnagle, M. LeGendre, M.**
596   **Hurley, J. Fantone, and C. Engleberg.** 1996. Coinoculation with *Hartmannella*
597   *vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine
598   model of Legionnaires' disease. Infect. Immun. **64:**2449-2456.
599   8.   **Chaudhuri, R. R., C. P. Ren, L. Desmond, G. A. Vincent, N. J. Silman, J. K. Brehm,**
600   **M. J. Elmore, M. J. Hudson, M. Forsman, K. E. Isherwood, D. Gurycova, N. P.**
601   **Minton, R. W. Titball, M. J. Pallen, and R. Vipond.** 2007. Genome sequencing shows
602   that European isolates of *Francisella tularensis* subspecies t*ularensis* are almost identical
603   to US laboratory strain Schu S4. PLoS ONE **2:**e352.
604   9.   **Chisholm, G. E. t., and M. H. Vaughan.** 1979. Isolation and characterization of a
605   cycloheximide-resistant mutant of *Acanthamoeba castellanii* Neff. J. Bacteriol. **138:**280-
606   283.
607   10.   **Cirillo, J. D., S. L. Cirillo, L. Yan, L. E. Bermudez, S. Falkow, and L. S. Tompkins.**
608   1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry
609   mechanisms and enhances virulence of *Legionella pneumophila.* Infect Immun **67:**4427-
610   4434.
611   11.   **Cirillo, J. D., S. Falkow, and L. S. Tompkins.** 1994. Growth of *Legionella*
612   *pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infect. Immun. **62:**3254-
613   3261.
614   12.   **Cirillo, J. D., S. Falkow, L. S. Tompkins, and L. E. Bermudez.** 1997. Interaction of
615   *Mycobacterium avium* with environmental amoebae enhances virulence. Infect. Immun.
616   **65:**3759-3767.
617   13.   **Cirillo, S. L., L. E. Bermudez, S. H. El-Etr, G. E. Duhamel, and J. D. Cirillo.** 2001.
618   *Legionella pneumophila* entry gene *rtxA* is involved in virulence. Infect. Immun. **69:**508-
619   517.
620   14.   **Cirillo, S. L., L. Yan, M. Littman, M. M. Samrakandi, and J. D. Cirillo.** 2002. Role
621   of the *Legionella pneumophila rtxA* gene in amoebae. Microbiology **148:**1667-1677.

26

622 15. **Darling, R. G., C. L. Catlett, K. D. Huebner, and D. G. Jarrett.** 2002. Threats in
623 bioterrorism. I: CDC category A agents. Emerg. Med. Clin. North. Am. **20:**273-309.
624 16. **Eigelsbach, H. T., W. Braun, and R. D. Herring.** 1951. Studies on the variation of
625 *Bacterium tularense.* J. Bacteriol. **61:**557-569.
626 17. **Eigelsbach, H. T., and C. M. Downs.** 1961. Prophylactic effectiveness of live and killed
627 tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and
628 guinea pig. J. Immunol. **87:**415-425.
629 18. **El-Etr, S. H., A. Mueller, L. S. Tompkins, S. Falkow, and D. S. Merrell.** 2004.
630 Phosphorylation-independent effects of CagA during interaction between Helicobacter
631 pylori and T84 polarized monolayers. J Infect Dis **190:**1516-1523.
632 19. **El-Etr, S. H., L. Yan, and J. D. Cirillo.** 2001. Fish monocytes as a model for
633 mycobacterial host-pathogen interactions. Infect. Immun. **69:**7310-7317.
634 20. **Eliasson, H., and E. Back.** 2007. Tularaemia in an emergent area in Sweden: an analysis
635 of 234 cases in five years. Scand. J. Infect. Dis. **39:**880-889.
636 21. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin. Microbiol.
637 Rev. **15:**631-646.
638 22. **Gonzalez, J., G. Bai, U. Frevet, E. J. Corey and D. Eichinger.** 1999. Proteasome-
639 dependent cyst formation and stage-specific ubiquitin mRNA accumulation in
640 *Entamoeba invadens.* Eur. J. Biochem. **264:**897-904.
641 23. **Greub, G., B. La Scola, and D. Raoult.** 2003. *Parachlamydia acanthamoeba* is
642 endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation
643 temperature. Ann. N Y Acad. Sci. **990:**628-634.
644 24. **Greub, G., and D. Raoult.** 2004. Microorganisms resistant to free-living amoebae. Clin.
645 Microbiol. Rev. **17:**413-433.
646 25. **Gurcan, S., M. Eskiocak, G. Varol, C. Uzun, M. Tatman-Otkun, N. Sakru, A.**
647 **Karadenizli, C. Karagol, and M. Otkun.** 2006. Tularemia re-emerging in European
648 part of Turkey after 60 years. Jpn. J. Infect. Dis. **59:**391-393.
649 26. **Hernandez, L. D., K. Hueffer, M. R. Wenk, and J. E. Galan.** 2004. *Salmonella*
650 modulates vesicular traffic by altering phosphoinositide metabolism. Science **304:**1805-
651 1807.
652 27. **Kaiser, D. A., V. K. Vinson, D. B. Murphy, and T. D. Pollard.** 1999. Profilin is
653 predominantly associated with monomeric actin in *Acanthamoeba.* J. Cell Sci. **112 (Pt**
654 **21):**3779-3790.
655 28. **Khunkitti, W., D. Lloyd, J. R. Furr, and A. D. Russell.** 1998. *Acanthamoeba*
656 *castellanii*: growth, encystment, excystment and biocide susceptibility. J Infect **36:**43-48.
657 29. **Kilvington, S., W. Heaselgrave, J. M. Lally, K. Ambrus, and H. Powell.** 2008.
658 Encystment of *Acanthamoeba* during incubation in multipurpose contact lens disinfectant
659 solutions and experimental formulations. Eye Contact Lens. **34:**133-1399.
660 30. **Lloyd, D., N. A. Turner, W. Khunkitti, A. C. Hann, J. R. Furr, and A. D. Russell.**
661 2001. Encystation in *Acanthamoeba castellanii*: development of biocide resistance. J.
662 Eukaryot. Microbiol. **48:**11-16.
663 31. **Lundman, T.** 2005. Watch out for tularemia also in Southern Sweden! Lakartidningen
664 **102:**1986-1987.
665 32. **McCoy, G. W. a. C. W. C.** 1912. Further observations of a plague-like disease of
666 rodents with a preliminary note of the causative agent, *Bacterium tularensis.* J. Infect.
667 Dis. **10:**61-72.

668 33. **Moon, E. K., D. I. Chung, Y. C. Hong, T. I. Ahn, and H. H. Kong.** 2008.
669 *Acanthamoeba castellanii*: gene profile of encystation by ESTs analysis and KOG
670 assignment. Exp. Parasitol. **119:**111-116.
671 34. **Moon, E. K., D. I. Chung, Y. C. Hong, and H. H. Kong.** 2008. Characterization of a
672 serine proteinase mediating encystation of *Acanthamoeba.* Eukaryot. Cell **7:**1513-1517.
673 35. **Morner, T.** 1992. The ecology of tularaemia. Rev. Sci. Tech. **11:**1123-1130.
674 36. **Oyston, P. C.** 2008. *Francisella tularensis*: unravelling the secrets of an intracellular
675 pathogen. J. Med. Microbiol. **57:**921-930.
676 37. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence
677 renews interest in *Francisella tularensis*. Nat. Rev. Microbiol. **2:**967-978.
678 38. **Pagano, M.** 1997. Cell cycle regulation by the ubiquitin pathway. Faseb J **11:**1067-1075.
679 39. **Paoletti, M., M. Castroviejo, J. Begueret, and C. Clave.** 2001. Identification and
680 characterization of a gene encoding a subtilisin-like serine protease induced during the
681 vegetative incompatibility reaction in *Podospora anserina.* Curr. Genet. **39:**244-252.
682 40. **Parker, R. R., E. A. Steinhaus, G. M. Kohls, and W. L. Jellison.** 1951. Contamination
683 of natural waters and mud with *Pasteurella tularensis* and tularemia in beavers and
684 muskrats in the northwestern United States. Bull. Natl. Inst. Health **193:**1-161.
685 41. **Penn, R. L.** 2005. *Francisella tularensis* (tularemia). vol. 1. Churchill Livingstone, New
686 York.
687 42. **Petersen, J. M., and M. E. Schriefer.** 2005. Tularemia: emergence/re-emergence. Vet.
688 Res. **36:**455-467.
689 43. **Santic, M., M. Molmeret, K. E. Klose, S. Jones, and Y. A. Kwaik.** 2005. The
690 *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are
691 essential for modulating phagosome biogenesis and subsequent bacterial escape into the
692 cytoplasm. Cell. Microbiol. **7:**969-979.
693 44. **Saslaw, S., and S. Carhart.** 1961. Studies with tularemia vaccines in volunteers. III.
694 Serologic aspects following intracutaneous or respiratory challenge in both vaccinated
695 and nonvaccinated volunteers. Am. J. Med. Sci. **241:**689-699.
696 45. **Saslaw, S., H. T. Eigelsbach, J. A. Prior, H. E. Wilson, and S. Carhart.** 1961.
697 Tularemia vaccine study. II. Respiratory challenge. Arch. Intern. Med. **107:**702-714.
698 46. **Saslaw, S., H. T. Eigelsbach, H. E. Wilson, J. A. Prior, and S. Carhart.** 1961.
699 Tularemia vaccine study. I. Intracutaneous challenge. Arch. Intern. Med. **107:**689-701.
700 47. **Sata, T.** 2005. Bioterrorism. Nihon. Hoigaku. Zasshi. **59:**119-125.
701 48. **Schuster, F. L.** 2002. Cultivation of pathogenic and opportunistic free-living amebas.
702 Clin. Microbio.l Rev. **15:**342-354.
703 49. **Sjostedt, A.** 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical
704 manifestations. Ann. NY Acad. Sci. **1105:**1-29.
705 50. **Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi.** 1992. Autophagy in
706 yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J.
707 Cell Biol. **119:**301-311.
708 51. **Vandekerckhove, J. S., D. A. Kaiser, and T. D. Pollard.** 1989. *Acanthamoeba* actin
709 and profilin can be cross-linked between glutamic acid 364 of actin and lysine 115 of
710 profilin. J. Cell Biol. **109:**619-626.
711 52. **Wang, X., and D. G. Ahearn.** 1997. Effect of bacteria on survival and growth of
712 *Acanthamoeba castellanii.* Curr. Microbiol. **34:**212-215.

713   53.   **Weber, A.** 2004. Current epidemiology of selected bacterial zoonoses.
714   Gesundheitswesen **66 Suppl 1:**S26-30.
715   54.   **Yan, L., R. L. Cerny, and J. D. Cirillo.** 2004. Evidence that hsp90 is involved in the
716   altered interactions of *Acanthamoeba castellanii* variants with bacteria. Eukaryot. Cell
717   **3:**567-578.

718
719
720

721

722

723

724

725

726

727

728

729

730

731

732

733

734

735

736

737

738

739

FIGURE LEGENDS

741   **FIG. 1.** Cell association (A), entry (B) and intracellular survival (C) of *F. tularensis* strains

742   in *A. castellanii*. Cell association (A) and entry of LVS (B) were arbitrarily set at 1. The

743   intracellular survival rate (C) is represented as the ratio of CFU recovered at 24 hr to those

744   recovered at time zero for each strain. Data points and error bars represent the means and

745   standard deviations, respectively, of assays done in triplicate from representative experiments.

746   (** $P \leq 0.02$ and* $P \leq 0.05$)

747

748   **FIG. 2.** Growth curves of *F. tularensis* strains in PYG media (A) and Mueller Hinton broth

749   (B) at OD_{600} over a 24 hr time period. Data points and error bars represent the means and

750   standard deviations, respectively, of assays done in triplicate from a representative experiment.

751

752   **FIG. 3.** Transmission electron micrographs of *A. castellanii* infected with *F. tularensis*

753   strains. (A) Ft-1 is present in spacious vacuoles while LVS (B) is present in tight vacuoles at 2

754   hr post infection, long black arrows point to spacious vacuoles and short white arrows to tight

755   vacuoles . (B) LVS tight vacuole fused with nanogold labeled lysosomes at 2 hr post infection,

756   short white arrows point to nanogold particles. (C) Early cyst of *A. castellanii* containing Schu

757   S4 bacteria at 24 hr post infection. (D) *A. castellanii* trophozoite with multiple Schu S4 vacuoles

758   at 24 hr post infection. Bacterial vacuoles from (D) enlarged to show mitochondrial and

759   endoplasmic reticulum (white arrows) recruitment to the phagosome (E), and intact phagosomal

760   membranes (F). (b: bacteria, m: mitochondria).

761

762   **FIG. 4.** Immunofluorescence Z-projections of *A. castellanii* trophozoites (A) infected with

763   LVS expressing GFP for 2 hr (a, b and c) and novicida stained with an anti-*F. tularensis*

764   antibody (green) (f, g and h) after 2 hr and 30 min respectively. At 2 hr post infection the

765   majority of LVS (a) colocalizes with LysoTracker red (c) which appears diffuse within the

766   trophozoite (b). The majority of novicida (f) does not colocalize with LysoTracker red (h) which

767   appears localized (g). Enlarged cross-sections of panels (c) and (h) represent trophozoites

768   infected with LVS (d) and novicida (i) respectively. These were rotated by 90° using Volocity

769   software to confirm presence of the bacteria intracellularly (e and J).

770

771   **FIG. 5.** Nomarski DC Z-projections of uninfected *A. castellanii* cysts (e and f) and cysts

772   infected with novicida (a and b) and stained with an anti novicida antibody (b and f). An

773   enlarged cross-section of panels (b and f) was rotated by 90° using Volocity software to confirm

774   presence (d) or absence (h) of intracellular bacteria.

775

776   **FIG. 6.** (A) Rapid encystment of *A. castellanii* in response to infection with virulent *F.*

777   *tularensis* strains, LVS and novicida. Uninfected *A. castellanii* trophozoites (A). *A. castellanii*

778   trophozoites infected with LVS and Ft-3 (B and D respectively) for 2 hr. *A. castellanii* infected

779   with novicida, Schu, Ft-7, Ft-8 and Ft-10 (C, E, F, G and H respectively) or 2 hr. Arrows point

780   to early cysts and asterisks point to trophozoites.

781

782   **FIG. 7.** Quantification of the encystment of *A. castellanii* at 2 hr post infection with *F.*

783   *tularensis* strains. Data points and error bars represent the means and standard deviations

784   respectively of 3 random field counts of assays done in triplicate from representative

785   experiments (** $P \leq 0.02$, and * $P = 0.05$).

786

787   **FIG. 8.** Quantification of the encystment of naïve *A. castellanii* trophozoites, 4 hr after the

788   addition of fractions from *F. tularensis- A. castellanii* co-cultures. Data points and error bars

789   represent the means and standard deviations respectively of 3 random field counts of assays done

790   in triplicate from representative experiments (*$P \leq 0.02$).

791

792

793

794

795

796

797

798

799

800

801

802

803

804

805

806
807
808

TABLE 1. *F. tularensis* strains used in this study

Strain	Designation	Subtype	Region	Source	Year Isolated
LVS	LVS[b]	B	Europe	Sheep	1949, 1961
Novicida U112	NOV	NA	Utah	Human	1951
SCHU S4	SCHU[c]	A	Ohio	Human lesion	1941
70102163	Ft-1	A[a]	Utah	Human Blood	2001
79101574	Ft-2	A[a]	Utah	Human	1991
1365	Ft-3	A[a]	New Mexico	Human	1997
AS1284	Ft-4	A[a]	New Mexico	Rodent	2003
79400960	Ft-5	A[a]	Utah	Human	1990
80700069	Ft-6	A[a]	Utah	Human lesion	2007
80502541	Ft-7	A[a]	Utah	Human lesion	2005
1385	Ft-8	A[a]	New Mexico	Rabbit	2001
1773a	Ft-9	A[a]	New Mexico	Human	1999
AS2058	Ft-10	A[a]	New Mexico	Rabbit	2002

[a] Subtyping done using primers in IS100 elements unique to type A strains (Victoria Lao, Patrick Chain and Emilio Garcia, unpublished results).

[b] Live Vaccine Strain was produced as a vaccine in 1961 by passaging a virulent *F. t. holarctica* strain isolated in 1949 (17).

[c] Laboratory passaged strain.

Table 2. Quantification of *F. tularensis* in Infected *A. castellanii.*

Time	% tight vacuoles[a]				% tight vacuole fusion[b] (P)[e]	% Lysosomal Fusion[c]		(P)[f]
	LVS	NOV	SCHU	Ft-1		LVS	NOV	
30 m	ND[d]	ND	ND	ND	ND	48 ± 3	26 ± 2	0.02
2 hr	40 ± 3	10 ± 3	8 ± 2	9 ± 3	82 ± 2 (0.02)	89 ± 4	43 ± 3	0.01
24 hr	46 ± 2	9 ± 2	8 ± 3	10 ± 3	88 ± 4 (0.01)	ND	ND	ND

[a] Percentage of tight bacterial vacuoles in cells containing at least one bacterial vacuole. Results are the means ± standard deviations of two counts of 50 cells in different sections of two separate preparations.

[b] Co-localization of nanogold particles with bacterial vacuoles from all *F. tularensis* strains processed. Results are the means ± standard deviations of two counts of 50 cells containing at least one bacterial vacuole in different sections of two separate preparations.

[c] Co-localization of LysoTracker red with bacterial vacuoles containing at least one bacterium by IF. Results are the means ± standard deviations of two counts of 25 cells from 4 separate preparations.

[d] ND: Not Done, sample too small.

[e] P values indicate the significance of fusion of *F. tularensis* tight vacuoles with nanogold particles.

[f] P values indicate the significance of co-localization of novicida and LVS vacuoles with LysoTracker red.

TABLE 3. Survival of *F. tularensis* strains in *A. castellanii.*

Time (days)	LVS		NOV		SCHU		Ft-1		Ft-7		UI[a]
	- c	+ c[b]	- c	+ c	- c	+ c	- c	+ c	- c	+ c	- c
3	+[c]	NG[d]	+	NG	+	NG	+	NG	+	NG	NG
7	NG	ND[e]	+	NG	+	NG	+	NG	+	NG	NG
14	NG	ND	+	NG	+	ND	+	ND	+	ND	NG
21	NG	ND	+	ND	NG	ND	+	ND	+	ND	NG

[a] Uninfected *A. castellanii* control.

[b] *A. castellanii* cysts treated with (+ c) or without (- c) 25μg/ml cycloheximide prior to addition of HS buffer to prevent excystment.

[c] (+): Turbidity in experimental wells 48-72 hr after replacing buffer with rich media. *F. tularensis* growth confirmed by plating for viable CFU and gram staining. In all cases where turbidity was observed >10^8 CFU/ml were present.

[d] NG: No growth after replacing buffer with rich medium and plating for viable CFU.

[e] ND: Not Done

TABLE 4. Survival of *F. tularensis* strains in *A. castellanii* in the presence and absence of encystment.

Time (days)	SCHU		Ft-1		NOV		LVS	
	- c	+ c[a]	-c	+ c	-c	+ c	R	+ R[b]
3	+[c], E[d]	NG[e], NE[f]	+, E	NG, NE	+, E	NG, NE	+	+, E
8	+, E	NG, NE	+, E	NG, NE	+, E	NG, NE	NG	+, E
14	+, E	ND[g]	+, E	ND	+, E	ND	NG	NG

[a] *A. castellanii* trophozoites treated with (+ c) or without (- c) 25μg/ml cycloheximide to prevent encystment.

[b] *A. castellanii* infected with LVS in the presence (+ R) or absence (- R) of the REP fractions from novicida -*A. castellanii* or Schu S4- *A. castellanii* co-cultures.

[c] (+): Turbidity in experimental wells 48-72 hr after replacing buffer with rich media. *F. tularensis* growth confirmed by plating for viable CFU and gram staining. In all cases where turbidity was observed >10^8 CFU/ml were present.

[d] E: At least 50% of *A. castellanii* present are encysted (E).

[e] NG: No growth after replacing buffer with rich medium and plating for viable CFU.

[f] NE: Less than 10% of *A. castellanii* present are encysted.

[g] ND: Not Done.

TABLE 5: Identification of *A. castellanii* Proteins in the REP Fraction.

	Protein	Locus	Size (kD)	No. of Peptides[a]
922				
923	Ubiquitin/	P49634	15.4	4
924	Ubiquitin Fusion Protein	CAA53293	194	4
925	Profilin II, α chain	P19984	21.9	2
926	Actin	P02578	29.8	2
927	Encystation mediating			
928	Serine proteinase/	ABY63398	32.1	16
929	Subtilisin-like Serine			
930	Proteinase	AAF91465	16.5	7
931	DnaK molecular chaperone/	AAU94654	37.5	2
933	HSP 70 family protein			

934   [a] Number of peptides sequenced by Nano LC-MS/MS.

935

XY

YZ