

Survival and Growth of *Francisella tularensis* in *Acanthamoeba castellanii*

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Francisella tularensis is a highly infectious, facultative intracellular bacterium which causes epidemics of tularemia in both humans and mammals at regular intervals. The natural reservoir of the bacterium is largely unknown, although it has been speculated that protozoa may harbor it. To test this hypothesis, *Acanthamoeba castellanii* was cocultured with a strain of *F. tularensis* engineered to produce green fluorescent protein (GFP) in a nutrient-rich medium. GFP fluorescence within *A. castellanii* was then monitored by flow cytometry and fluorescence microscopy. In addition, extracellular bacteria were distinguished from intracellular bacteria by targeting with monoclonal antibodies. Electron microscopy was used to determine the intracellular location of *F. tularensis* in *A. castellanii*, and viable counts were obtained for both extracellular and intracellular bacteria. The results showed that many *F. tularensis* cells were located intracellularly in *A. castellanii* cells. The bacteria multiplied within intracellular vacuoles and eventually killed many of the host cells. *F. tularensis* was found in intact trophozoites, excreted vesicles, and cysts. Furthermore, *F. tularensis* grew faster in cocultures with *A. castellanii* than it did when grown alone in the same medium. This increase in growth was accompanied by a decrease in the number of *A. castellanii* cells. The interaction between *F. tularensis* and amoebae demonstrated in this study indicates that ubiquitous protozoa might be an important environmental reservoir for *F. tularensis*.

Francisella tularensis, a gram-negative, nonsporulating, nonmotile, and capsulated coccobacillus, is a facultative intracellular bacterium which causes tularemia in humans and animals (37). Inoculation or inhalation of as few as 10 *F. tularensis* organisms may cause disease in humans (35, 36), and because of its high virulence and contagiousness, the bacterium is considered a possible bioterrorism agent (7). It has been isolated from approximately 250 wildlife species (37), but its principal natural reservoir is largely unknown, although studies have shown that the organism may persist for more than a year in water or mud (30).

Virtually nothing is known about the virulence mechanisms of *F. tularensis* or about how it survives within host cells. Even the avirulent live vaccine strain (LVS), which has been used as a human vaccine (34), is strongly pathogenic for certain animals and causes a lethal infection in mice that is indistinguishable from human disease (8). It has been shown that LVS is very well adapted to the intracellular environment of macrophages, survives in phagosomes (3, 13), and exerts a cytopathogenic effect on murine macrophages (3a, 5, 13). Furthermore, it has been found that LVS localizes within an acidic vesicle, which facilitates its iron uptake (11), and that LVS releases an acid phosphatase that inhibits the respiratory burst in neutrophils (33). Recently, it has also been demonstrated that infec-

tion with the LVS strain induces apoptosis in murine macrophages (24).

Free-living amoebae such as *Acanthamoeba* species are commonly found in natural aquatic systems (25). As a part of biofilms in aquatic environments, free-living amoebae and bacteria are involved in complex interactions. *Francisella* species are also known to be connected with natural water systems (18, 30), and it is well known that *Acanthamoeba* species are environmental hosts of several intracellular pathogens, such as *Legionella*, *Chlamydia*, and *Mycobacterium* (2, 26, 38, 39). *Legionella* cells interact with their protozoan hosts and mammalian cells in a similar way. The known genetic factors required by *Legionella* to infect protozoa are also required for the infection process in mammalian cells (16, 17). Previous studies of the interaction between *F. tularensis* and *Acanthamoeba castellanii* have shown that growth of *F. tularensis* is enhanced in media preconditioned by amoebae (15). Here, we have tested the hypothesis that protozoa may comprise a significant environmental reservoir for *F. tularensis*.

MATERIALS AND METHODS

Microorganisms and plasmid construction. *A. castellanii* (ATCC 30234) was obtained from the American Type Culture Collection (ATCC), Manassas, Va., and *F. tularensis* LVS (type B) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. *F. tularensis* LVS carrying a destabilized form of green fluorescent protein (GFP) was constructed as follows. First, the promoter for GroEL from *F. tularensis* LVS (9) was amplified by PCR, cloned into the *SmaI-PstI* sites upstream of the chloramphenicol acetyltransferase (CAT) gene in pKK214 (23, 29), and then introduced into *F. tularensis* LVS by cryotransformation (31). The resulting plasmid was designated pKK214CAT and was used as the basic construct in the present work. A mutant form (M2) of wild-type GFP (6) was amplified by PCR and cloned into the *PstI-EcoRI* sites of the pKK214CAT plasmid under the control of the GroEL promoter. Because the *EcoRI* site is inside the CAT gene, this procedure led to

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the partial removal of the CAT gene. The resulting plasmid was designated pKK214GFP. Next, a destabilized variant (ASV) of GFP (Clontech, Palo Alto, Calif.) was amplified by PCR and cut with *Bsp1407I-EcoRI*, and the resulting *Bsp1407I-EcoRI* fragment was cloned into pKK214GFP cut with the same enzymes. This resulted in substitution of the C-terminal part of GFP (M2 form) for the destabilized C-terminal form (ASV), which contains sequences encoding protease targets responsible for the rapid degradation of the expressed protein. This construct was designated pKK214GFP/ASV. To determine the half-life of this GFP/ASV construction in *F. tularensis* LVS, samples containing 2.0×10^9 CFU/ml were inactivated by treatment with 250 μ g of gentamicin (Sigma, St. Louis, Mo.) per ml for 1 h at room temperature in darkness. After confirming that the cells were unable to grow, as determined by viable counts, following this treatment, viable count and flow cytometry analyses were carried out in parallel for 7 days. No growth on modified Thayer-Martin agar was observed, and the fluorescence gradually decreased.

Culture media and growth conditions. *A. castellanii* was grown without shaking at 30°C to a final concentration of 10^6 /ml in ATCC medium no. 712 (ATCC). Modified Thayer-Martin agar plates containing 36 g of GC base medium (Difco Laboratories, Detroit, Mich.) per liter, 10 g of hemoglobin (Difco) per liter, 10 mg of IsoVitalX (BBL Microbiology Systems, Cockeysville, Md.) per liter, and 10 mg of tetracycline (Sigma) per liter were used for viable counts and growth of *F. tularensis* LVS/GFP/ASV. After the inoculum was spread as serial dilutions, the agar plates were incubated at 37°C in 5% CO₂ for at least 2 days if not otherwise stated. Cocultures of *F. tularensis* LVS/GFP/ASV and *A. castellanii* were incubated in Falcon tissue culture flasks (75 cm²) filled with 50 ml of ATCC medium 712 containing 20 μ g of tetracycline per ml and an initial concentration of 10^5 *A. castellanii* cells and 10^6 *F. tularensis* cells per ml. Control flasks with the microorganisms cultured separately were prepared in the same way and with the same initial concentrations. The flasks were incubated at 30°C without shaking. Samples were withdrawn regularly for electron microscopy, flow cytometry, and fluorescence microscopy analysis and to obtain viable counts.

Flow cytometry analysis. The flow cytometer (FACSort; Becton Dickinson Immuno Systems, San Jose, Calif.), equipped with an argon laser giving a 488-nm primary emission line, was calibrated with unlabeled and labeled beads (Becton Dickinson) and FACSComp software (Becton Dickinson). Unlabeled cells were adjusted for forward scatter (relative size), side scatter (relative granularity), FL1 (green color), FL2 (red color), and FL3 (deep red color). The measuring time per sample was 15 to 50 s, with a medium flow rate of 60 μ l/min. From each sample 10,000 events were registered, and data were analyzed with Cell Quest software (Becton Dickinson). Samples (3 ml) of cell suspension from the coculture flasks were centrifuged for 10 min at 300 \times g in a model TJ6 centrifuge (Beckman Instruments, Palo Alto, Calif.) and washed six times with FacsWash solution (Becton Dickinson) prior to the analyses.

Microscopy analysis. *A. castellanii* cells were counted in a Bürker counting chamber (Merck Eurolab, Stockholm, Sweden) under a light microscope (Leica Microscopy Systems, Heerburg, Switzerland), while the abundance and distribution of *F. tularensis* LVS/GFP/ASV were analyzed by fluorescence microscopy (Leica Microscopy Systems). Prior to analysis, using an oil immersion lens and a green filter, 2-ml samples of cell suspension from the coculture flask were centrifuged for 10 min at 300 \times g in a Beckman model TJ6 centrifuge, and the resulting pellets were washed six times with FacsWash solution.

Antibody labeling of extracellular *F. tularensis* LVS/GFP/ASV was performed by adding 10 μ l of biotin-labeled antibodies, specific for *F. tularensis* LVS (German Armed Forces Medical Academy, Munich, Germany), to 1-ml samples of cell suspension from coculture flasks. The samples were incubated for 20 min at room temperature, washed, reincubated for 20 min with 10 μ l of R-phycoerythrin-conjugated Streptavidin (DAKO, Glostrup, Denmark), washed again, and examined under the fluorescence microscope.

For electron microscopy, 2-ml samples of cell suspension from the coculture flasks were centrifuged for 10 min at 300 \times g in a Beckman model TJ6 centrifuge, and the resulting pellets were washed six times with FacsWash solution. Each pellet of infected amoebae was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h at room temperature. The samples were then washed twice in the same buffer and fixed in 1% reduced osmium tetroxide for 1 h. The specimens were centrifuged into pellets, which were embedded in a 2% solution of agarose at 45°C. The solidified agarose containing the amoebae was cut into small cubes, which were dehydrated in graded acetone and embedded in an Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (EM 900; Carl Zeiss, Oberkochen, Germany).

Analysis of viable *F. tularensis* within *A. castellanii*. Samples (3 ml) of cell suspensions from coculture flasks were centrifuged for 10 min at 300 \times g in a Beckman model TJ6 centrifuge and washed six times with FacsWash solution to

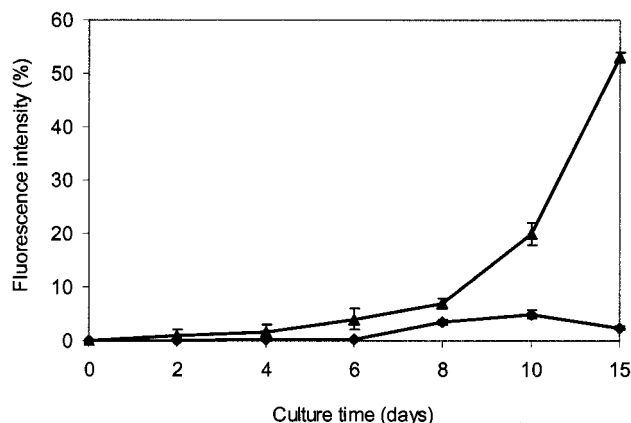


FIG. 1. Changes in GFP fluorescence over time in cell populations of *A. castellanii* infected with *F. tularensis* LVS/GFP/ASV (triangles) and autofluorescence from *A. castellanii* (squares), measured by flow cytometry. Mean values from two independent experiments are shown (*t* test, *P* < 0.05). Bars show averages of the absolute deviations of data points from their mean.

minimize extracellular *F. tularensis* contamination. The pellets were resuspended in 500 μ l of FacsWash solution and incubated with 250 μ g of gentamicin per ml for 1 h at room temperature. Next, the samples were centrifuged for 10 min at 300 \times g in a Beckman model TJ6 centrifuge, and 100- μ l portions of the supernatant were spread on modified Thayer-Martin agar plates. Each pellet was diluted twofold with 0.5% sodium deoxycholate and incubated for 5 min at room temperature, after which series of 10-fold dilutions from 10^1 to 10^4 were prepared and spread on modified Thayer-Martin agar.

RESULTS

GFP fluorescence from *F. tularensis* in *A. castellanii*. Prior to the analyses of GFP fluorescence from *F. tularensis* in *A. castellanii*, we determined the half-life of the destabilized form (ASV) of the GFP expressed in *F. tularensis* LVS/GFP/ASV. The half-life was found to be approximately 34 h. This ensured that viable cells were measured, since dying cells would show lower fluorescence intensity with time.

Flow cytometer analysis of samples taken from the flasks containing both *F. tularensis* LVS/GFP/ASV and *A. castellanii* showed that the fluorescence intensity increased with time in the *A. castellanii* population, from 0% at day 0 to 54% at day 15 (Fig. 1).

Intracellular location of *F. tularensis* in *A. castellanii*. Samples taken from cocultures of *F. tularensis* LVS/GFP/ASV and *A. castellanii* showed an increase in the numbers of intracellular bacteria with time (Fig. 2). Moreover, different stages of infection were observed, including growth in intracellular-membrane-limited vacuoles, release of vesicles, and cysts containing bacteria. Samples taken at day 18 from the coculture were mixed with R-phycoerythrin-labeled antibodies specific for *F. tularensis*, and the antibody-directed staining was visualized by fluorescence microscopy. The results showed that GFP-labeled bacteria within *A. castellanii* were not accessible to the antibodies and hence exhibited green fluorescence. In contrast, extracellular bacteria were bound by the antibodies and thus exhibited red fluorescence. This demonstrated that many of the *F. tularensis* cells were located intracellularly (Fig. 3). The fluorescence microscopy also showed that *F. tularensis*-infected amoebae disintegrated as vesicles inside them filled

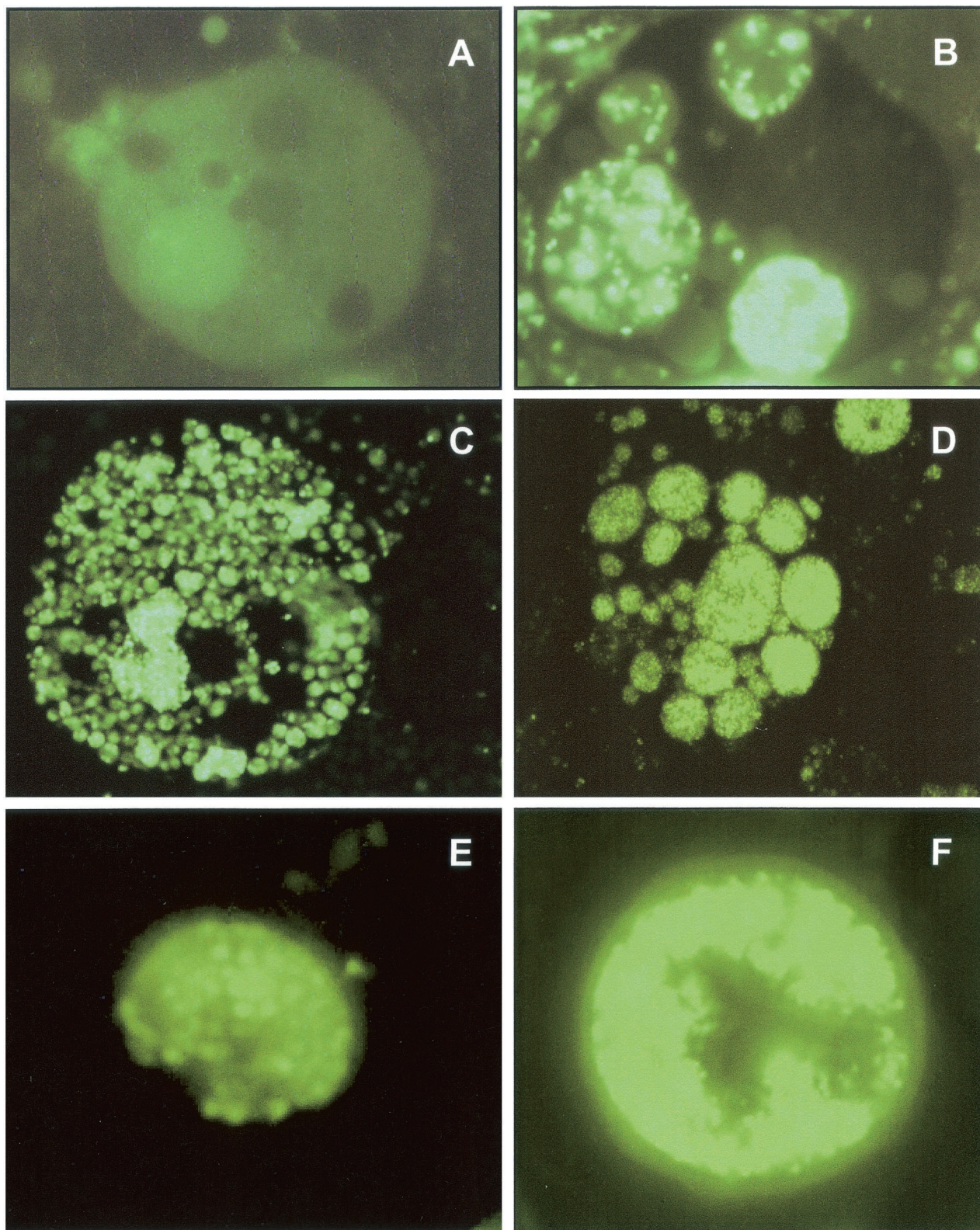


FIG. 2. Fluorescence microscopy analysis. (A) *A. castellanii* trophozoite without intracellular *F. tularensis* (day 0). (B) Intact *A. castellanii* with *Francisella*-filled vacuoles (day 10). (C) Disintegrating *A. castellanii* filled with *F. tularensis* LVS/GFP/ASV (day 15). (D) *Francisella*-filled vesicles, enclosed within the cell membrane of a dead *A. castellanii* trophozoite (day 18). (E) *Francisella*-filled vesicle (day 18). (F) *A. castellanii* cyst containing *F. tularensis* LVS/GFP/ASV inside the double wall (day 40).

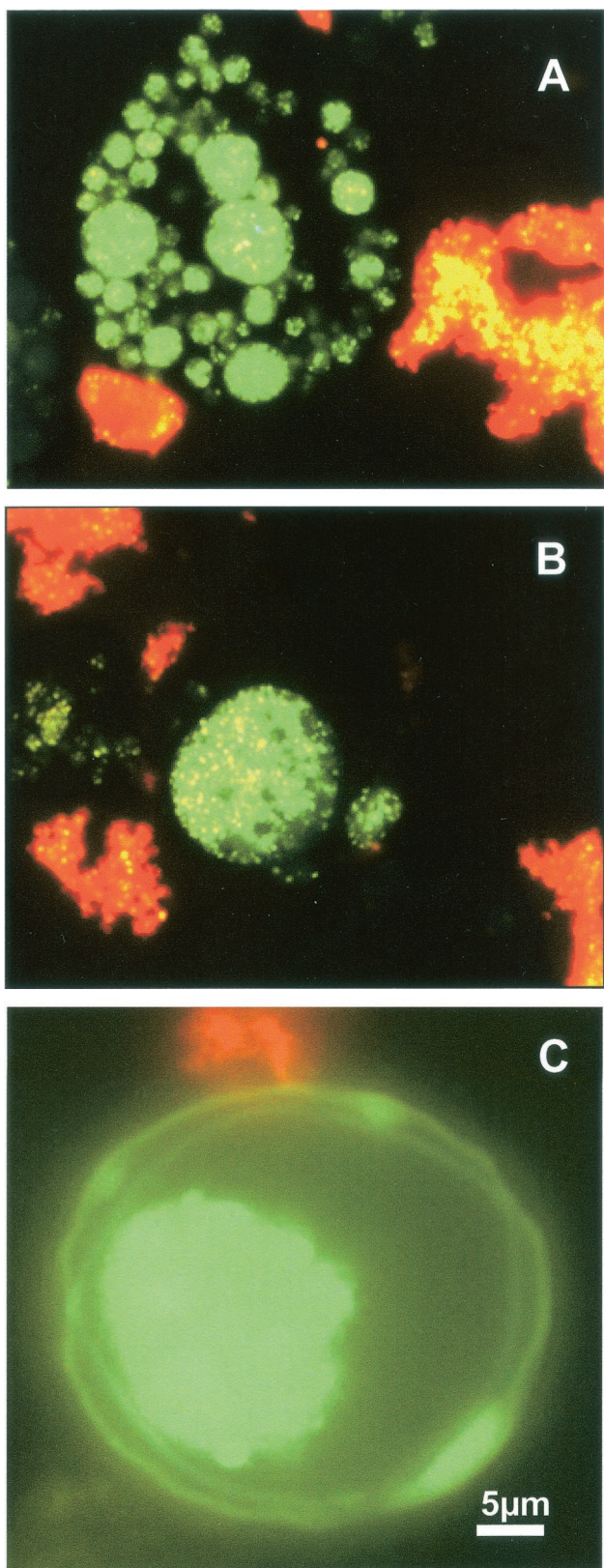


FIG. 3. Differentiation between extracellular and intracellular *F. tularensis* by use of monoclonal antibodies. Viable intracellular *F. tularensis* LVS/GFP/AVS cells expressing GFP appear green, while extracellular *F. tularensis* cells appear red after treatment with labeled antibodies specific for *F. tularensis*. (A) Disintegrating *A. castellanii*

with *F. tularensis* and that bacteria occurred within cysts (Fig. 2 and 3).

Electron micrographs confirmed that many *F. tularensis* cells were located within vacuoles in *A. castellanii* (Fig. 4). The vacuoles containing bacteria seemed to attract amoebal organelles such as mitochondria and rough endoplasmic reticulum (Fig. 4C and D). As shown in Fig. 4E, *F. tularensis* cells could be seen lining up between an emerging double wall, which is a sign of encystation. However, another outcome was also observed in some cases (Fig. 2F, 3C, and 4F), in which all the bacteria seemed to be located inside the double wall of the cyst.

Growth of cocultures of *F. tularensis* LVS/GFP/ASV and *A. castellanii*. Viable counts of extracellular *F. tularensis* LVS/GFP/ASV increased in the presence of *A. castellanii* from 2×10^6 CFU/ml at day 0 to 2×10^9 CFU/ml at day 15. In contrast, viable counts of *F. tularensis* LVS/GFP/ASV cultured alone decreased from 2×10^6 CFU/ml at day 0 to nondetectable levels at day 15 (Fig. 5). Differences between the numbers of *A. castellanii* cells in cultures with or without *F. tularensis* LVS/GFP/ASV were also apparent. The total counts, according to Bürker chamber determinations, showed that the numbers of *A. castellanii* cells increased from 4×10^5 cells/ml at day 0 to 8×10^5 cells/ml at day 20 when cultured alone and decreased from 4×10^5 cells/ml at day 0 to 3×10^5 cells/ml at day 20 when cocultured with *F. tularensis* LVS/GFP/ASV (Fig. 6).

The viability of the *F. tularensis* cells within *A. castellanii* was analyzed by taking viable counts of the bacteria after gentamicin treatment to kill extracellular bacteria followed by deoxycholate treatment of the *A. castellanii* cells to release the intracellular bacteria. No bacteria were recovered from gentamicin-treated medium, but in *A. castellanii* cells, an increase in *F. tularensis* LVS/GFP/ASV viable counts was observed over time, from 0 cells/ml at days 0, 2, 4, and 8 to 2×10^6 cells/ml at day 15 (Fig. 5).

DISCUSSION

F. tularensis is a highly infectious and facultative intracellular bacterium that causes various types of tularemia in mammals (18). It has been considered a potential biological weapon and possible bioterrorism agent (7), so its survival in nature is of interest from an epidemiological perspective. The main reservoir of the bacterium is not known, but it has been shown that other intracellular bacteria, such as *Legionella*, *Mycobacterium*, and *Chlamydia*, utilize protozoa as natural reservoirs (2, 26, 38). In this context, it has been shown that growth of *F. tularensis* is enhanced in media preconditioned by *A. castellanii* (15). It has also been speculated that *F. tularensis* may persist in *A. castellanii* (4).

In the present study, we therefore aimed to study the interaction between *F. tularensis* and *A. castellanii* more thoroughly. The results show for the first time that *F. tularensis* can survive and grow within *A. castellanii*. The infection process begins

trophozoite containing *Francisella*-filled vesicles (day 18). (B) Individual vesicle containing viable *F. tularensis* GFP/LVS/ASV (day 18). (C) *A. castellanii* cyst containing viable *F. tularensis* inside the cyst double wall (day 40).

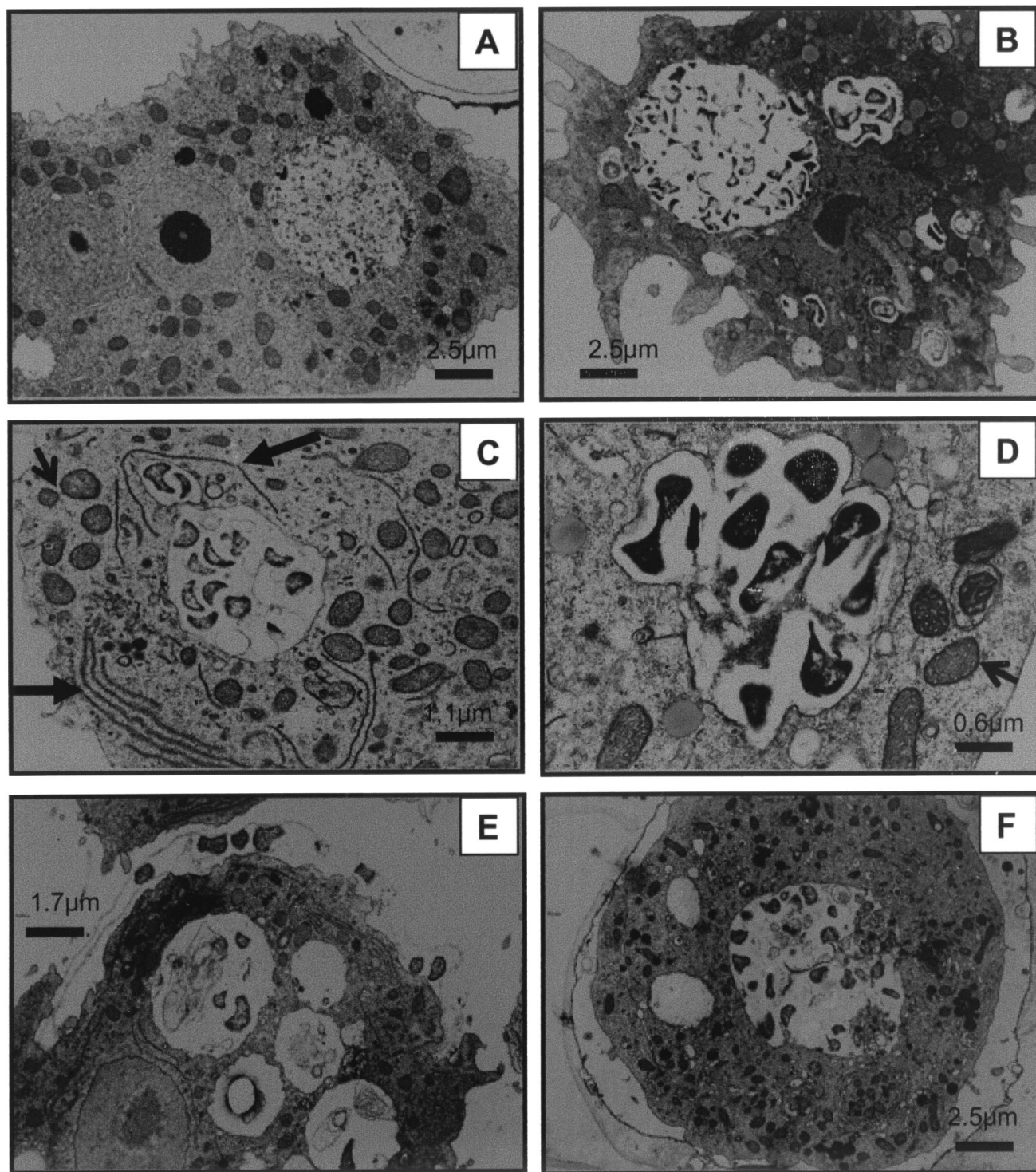


FIG. 4. Electron microscopy analysis. (A) *A. castellanii* trophozoite without intracellular *F. tularensis* (day 0). (B) *A. castellanii* trophozoite with *Francisella*-filled vacuoles (day 9). (C and D) Recruitment of mitochondria (short arrows) and rough endoplasmic reticulum (long arrows) to the vacuole containing bacteria. (E) *A. castellanii* trophozoite undergoing encystation with *F. tularensis* cells lined up between the two layers of the emerging double wall (day 16). (F) *A. castellanii* cyst containing *F. tularensis* on the inside of the double wall (day 16).

when trophozoites engulf *F. tularensis* cells, which replicate and grow in vacuole structures inside the trophozoites. Thus, the infection process in amoebae shows a resemblance to *Francisella* infection in macrophages (3, 11). Our ultrastructural

examinations showed that growth of *F. tularensis* in *A. castellanii* resulted in early changes in the amoebae, such as disappearance of the nucleus and condensation of the cytoplasm (Fig. 4). Moreover, cell organelles such as mitochondria and

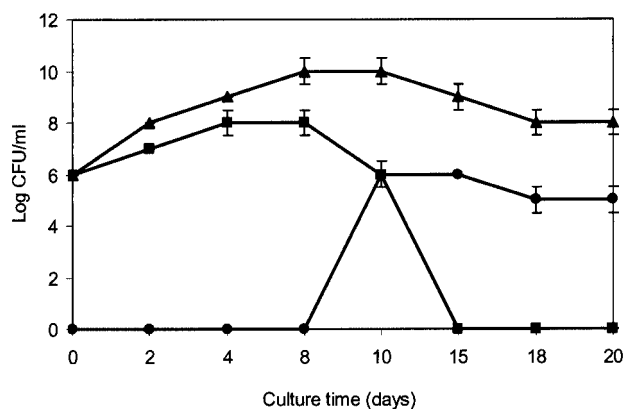


FIG. 5. Viable counts of *F. tularensis* cultured alone (squares), *F. tularensis* cocultured with *A. castellanii* (triangles), and intracellular *F. tularensis* (circles). Mean values from two independent experiments are shown (*t* test, $P < 0.05$). Bars show averages of the absolute deviations of data points from their mean.

endoplasmic reticulum were recruited to the vacuoles containing bacteria (Fig. 4C and D). This phenomenon has also been observed in *A. castellanii* infected with *Legionella* (1). Infected trophozoites were found both intact, filled with vacuoles containing *F. tularensis*, and in the process of cytolysis, excreting vesicles containing *F. tularensis*. Some infected trophozoites were also seen undergoing encystation, with two observed sequences of events: in one *F. tularensis* was contained inside the double wall, while in the other *F. tularensis* cells lined up in the space between the layers of the double wall (Fig. 3 and 4), as previously described for *Mycobacterium avium* and *Simkania negevensis* (22, 38). Thus, taking the observations together, the infection cycle of *F. tularensis* in *A. castellanii* seems to display many features in common with *Legionella* infection in *A. castellanii* (17).

Viable counts of cocultured *F. tularensis* showed that the presence of *A. castellanii* enhanced its growth. This finding is in accordance with previous results (15) and is probably due to the *F. tularensis* using CO_2 produced from live *A. castellanii* cells and nutrients derived from dead ones. In addition, intra-

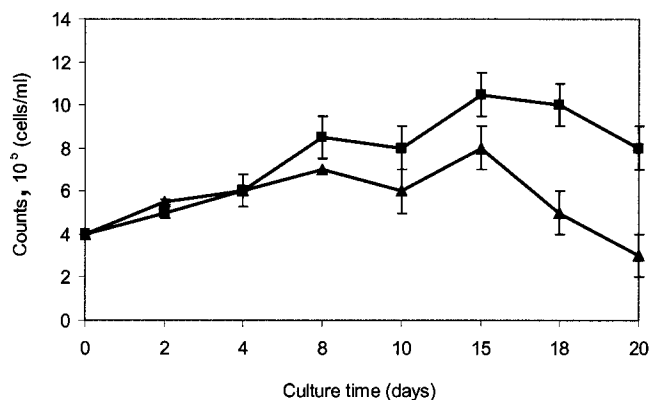


FIG. 6. Counts of *A. castellanii* cultured with *F. tularensis* (triangles) and without *F. tularensis* (squares) over time. Mean values from two independent experiments are shown (*t* test, $P < 0.05$). Bars show averages of the absolute deviations of data points from their mean.

cellular *F. tularensis* may escape into the culture medium after lysis of the *A. castellanii* cells.

Counts of *A. castellanii* cells showed that there were 25% fewer cells when they were cocultured with *F. tularensis* than when they were grown alone, apparently because *F. tularensis* killed a substantial number of them. Furthermore, the increase of fluorescence inside *A. castellanii* cells with time showed that the number of *F. tularensis* per amoeba increased over time. Accordingly, the viable counts of *F. tularensis* released from *A. castellanii* by deoxycholate treatment also increased over time.

In this study the attenuated vaccine strain *F. tularensis* LVS was used. Compared to virulent strains of *F. tularensis*, this strain obviously is not highly virulent for humans. However, it is still highly virulent for mice and guinea pigs (34). The genetic change(s) that results in attenuation is unknown. It is possible that the LVS strain could have a toxic effect on the protozoan host that is different from that of fully virulent *F. tularensis* strains.

Many types of bacteria have been found in free-living amoebae, including *Legionella* spp., *Burkholderia pickettii*, *Vibrio cholerae*, *Mycobacterium avium*, and *Listeria monocytogenes* (17, 39). These bacteria are able to survive inside the protozoa as endosymbionts and to take advantage of the amoebae as vectors for their dissemination. However, *Francisella* shows no close relationship to these genera, being most closely related to arthropod parasites and symbionts, including *Wolbachia persica*, the *Dermatocor andersoni* symbiont, and *Ornithodoros moubata* symbiont B (10, 27, 28). Other related strains, outside the *Francisellaceae*, include members of the *Piscirickettsiaceae*, in particular the intracellular bacterium *Piscirickettsia salmonis*, which is a fish pathogen (37).

Francisella bacteria are commonly transmitted to vertebrates via bites by ticks or mosquitoes (18, 19). However, ticks may not comprise a natural reservoir for *Francisella*, since infected ticks show a high mortality rate and transovarial transmission remains dubious (12, 19, 20). Moreover, the bacterium has been isolated from about 250 wildlife species (18, 37), clearly demonstrating that *F. tularensis* should be considered a highly successful intracellular parasite with a very broad host range and ability to survive and spread in widely differing natural environments.

The ability of *F. tularensis* to survive in trophozoites of *A. castellanii* and cysts, as demonstrated in this study, may have implications for the mode of transmission of the microorganism. However, although we have shown preliminary evidence for the survival of *F. tularensis* in amoebal cysts, the potential for its survival under various types of adverse conditions in these cysts still needs to be systematically examined. The close connection of tularemia with water (30) and the isolation of the bacterium from water samples used for domestic purposes, as well as from natural water systems, as the causal agent of outbreaks of the disease (14, 20, 21, 32) support the hypothesis that amoebae may have a role in the natural transmission of *F. tularensis*.

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