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Intracellular Growth of *Legionella pneumophila* Within *Acanthamoeba castellanii* Neff

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***Acanthamoeba castellanii* Neff supports the intracellular growth of *Legionella pneumophila*. When acanthamoebae were exposed to *L. pneumophila* for 1 h and then washed free of unassociated bacteria and placed in liquid culture, levels of viable amoeba-associated legionellae and legionellae free in the culture medium increased by three to four orders of magnitude in 48 to 72 h. However, most of the legionellae remained amoeba-associated and could be cultured only after disruption of the amoebae. Furthermore, legionella viability declined rapidly in amoeba culture medium alone or when bacteria and amoebae were separated by a microporous membrane. Therefore, direct amoeba-legionella contact is required for this growth. Infected acanthamoebae treated with cold acetone to permeabilize them to fluorescent-labeled anti-*L. pneumophila* antibody appeared to contain far more legionellae than amoebae fixed with glutaraldehyde so as to prevent antibody penetration. Electron micrographs of infected *A. castellanii* showed numerous bacteria, including some dividing forms, within vacuoles in the cytoplasm. These results together show that *A. castellanii* is able to provide an intracellular niche for the growth of *L. pneumophila*.**

The striking contrast between the fastidiousness of *Legionella pneumophila* under laboratory conditions (6) and the apparent ubiquity of these bacteria in aquatic environments (8, 9, 21) has led to a search for factors which may enhance legionella growth in natural habitats.

The observation of Tison et al. (20) of apparent legionella growth in association with blue-green algae in thermal environments has been extended in several laboratory studies of the interaction between these two types of organisms (2, 3, 16). In addition, Berendt (1) has reported the ability of algal extracts to enhance the aerosol stability of legionella, a critical factor in the spread of legionellosis.

The realization that small freshwater amoebae and legionellae are frequently found in similar thermal aquatic habitats led Rowbotham (17) to investigate the possible interactions between *L. pneumophila* and a variety of species of *Acanthamoeba* and *Naegleria*. In a series of agar plate experiments, he demonstrated that a heavy inoculum of *L. pneumophila* prevented the migration of these amoebae. Furthermore, by using light microscopy he found that amoebae which had come into contact with legionellae appeared to contain large numbers of these bacteria. When Nagington and Smith (14) cocultured *L. pneumophila* with the amoeba *Acanthamoeba polyphaga*, they found that the bacteria were so quickly consumed that viable legionellae were difficult to recover from the culture after 4 days.

Tyndall and Domingue (22) confirmed these observations by demonstrating that *Acanthamoeba royreba* and *Naegleria lovaniensis* could utilize legionellae as a sole food source. These authors also found that when *L. pneumophila* was cocultivated in suspension with either *A. royreba* or *N. lovaniensis*, the number of viable legionellae initially declined, then increased over an extended period even though the bacteria could not survive in the culture medium alone. This cocultivation caused no apparent change in the virulence status of either the legionellae or the amoebae based on intranasal inoculation of mice and intraperitoneal inoculation of guinea pigs.

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The studies described in this report were undertaken as a first step in defining the cell biology of the interaction between *L. pneumophila* and freshwater amoebae and the environmental implications thereof. In experiments similar to those of Tyndall and Domingue (22), we found that *L. pneumophila* grew rapidly when cocultured with the Neff strain of *Acanthamoeba castellanii*. These results suggested that either acanthamoebae produce an extracellular product which is utilized by legionellae for growth or *L. pneumophila* can grow intracellularly within these amoebae. Experiments employing parabiotic chambers for cultivation of amoebae and legionellae demonstrated the unlikelihood of a diffusible factor responsible for the growth of legionellae. The use of direct fluorescent antibody staining and of transmission electron microscopy identified the interior of the amoeba as the site where growth of *L. pneumophila* occurs. Therefore, we conclude that, at least under laboratory conditions, *L. pneumophila* is capable of infecting and multiplying within *A. castellanii* Neff.

MATERIALS AND METHODS

Organisms. The *L. pneumophila* Philadelphia-1 strain used in all experiments was first isolated on buffered charcoal yeast extract agar (BCYE) (6) from a guinea pig spleen homogenate obtained from the Centers for Disease Control, Atlanta, Ga., and was passaged no more than three times on BCYE agar before each experiment. The 50% lethal dose for this strain in male Hartley strain guinea pigs (400 to 450 g) was $>1.5 \times 10^7$ CFU per animal, with a 50% infective dose between 1.5×10^6 and 1.5×10^7 CFU per animal based on 2 consecutive days of fever of $\geq$38.9°C (13).

The well-characterized Neff strain of *A. castellanii* was obtained from the American Type Culture Collection (ATCC 30010) and was routinely cultivated as static monolayers at 30°C in acanthamoeba medium (AM; ATCC catalog of strains, medium 354). Subculture of amoeba stocks was performed routinely at intervals of ca. 2 weeks.

Cocultivation experiments. Axenic cultures of *A. castellanii* Neff were infected in AM with *L. pneumophila* at multiplicities of infection between 0.5 and 2.3. In a typical

experiment, a suspension of legionellae was prepared by suspending growth from a 46-h-old BCYE agar culture in 20 ml of sterile distilled water. A standard dilution containing ca. 10^8 *L. pneumophila* per ml based on turbidity was then prepared. This standard was then serially diluted in sterile distilled water and plated on BCYE agar to determine the concentration of viable legionellae.

Amoebae were enumerated by adding 0.1 ml of the culture to 0.8 ml of Page amoebal saline (PAS) (15) and 0.1 ml of trypan blue and then counting the number of trypan blue-impermeable trophozoites in a Fuchs-Rosenthal hemacytometer and bright-field illumination at ×10 magnification. All amoeba preparations were ≥95% viable by trypan blue exclusion.

Legionella-amoeba cultures (50-ml total volume) at the desired multiplicity of infection were incubated for 1 h at 30°C. Legionellae not associated with amoebae were removed by centrifuging the culture at 200 × *g* for 10 min. The supernatant was removed, and the pellet was resuspended in the same volume of fresh AM. This procedure was repeated four times, and after the final wash, the pellet was suspended to its original volume of 50 ml. The first and fourth supernatants removed during washing were serially diluted in sterile distilled water and plated on BCYE agar medium. The fourth wash supernatant routinely contained ≤0.1% of the total viable *L. pneumophila* initially added to the culture.

Immediately after washing and final suspension, a 10-ml sample was removed from the infected culture, and the remaining culture was incubated at 30°C. This 10-ml sample was first divided into two 5-ml samples, and the number of amoebae in each was determined. These 5-ml samples were then centrifuged at 200 × *g* for 10 min, and the supernatants were diluted in sterile distilled water and cultured on BCYE agar. One pellet was suspended in 5 ml of sterile distilled water, and the other was resuspended in 5 ml of PAS.

The washed amoebae suspended in sterile distilled water were disrupted by nitrogen cavitation at 1,000 lb/in^2 for 10 min, conditions shown by preliminary experiments to have no effect on the viability of *L. pneumophila*. The disrupted sample was serially diluted and plated on BCYE agar medium.

The amoebae suspended in 5 ml of PAS were placed on glass slides by using a Shandon-Southern Cytospin cytocentrifuge and then stained by the Gimenez technique (10). These slides were examined under bright-field illumination with a ×100 oil immersion objective, and the number of red-staining, rod-shaped bacteria associated with each of 200 amoebae were counted in each sample. Amoebae containing large numbers of densely packed bacteria were scored as containing 100 legionellae.

Additional 10-ml samples were removed from the infected amoeba cultures at later times and processed in the same manner. Controls consisting of amoebae which were not cocultivated with legionellae and legionellae added to AM alone were also assayed by the nitrogen cavitation procedure.

Culture of acanthamoebae and *L. pneumophila* in parabiotic chambers. The two compartments of a parabiotic chamber (membrane-type spinner flask, model 1972; Bellco Glass, Inc., Vineland, N.J.) were separated by a 0.4-μm-pore-size polycarbonate filter (Nuclepore Corp., Pleasanton, Calif.) and sealed with silicon lubricant. Chambers were sterilized by autoclaving and tested for leakage before the samples were loaded.

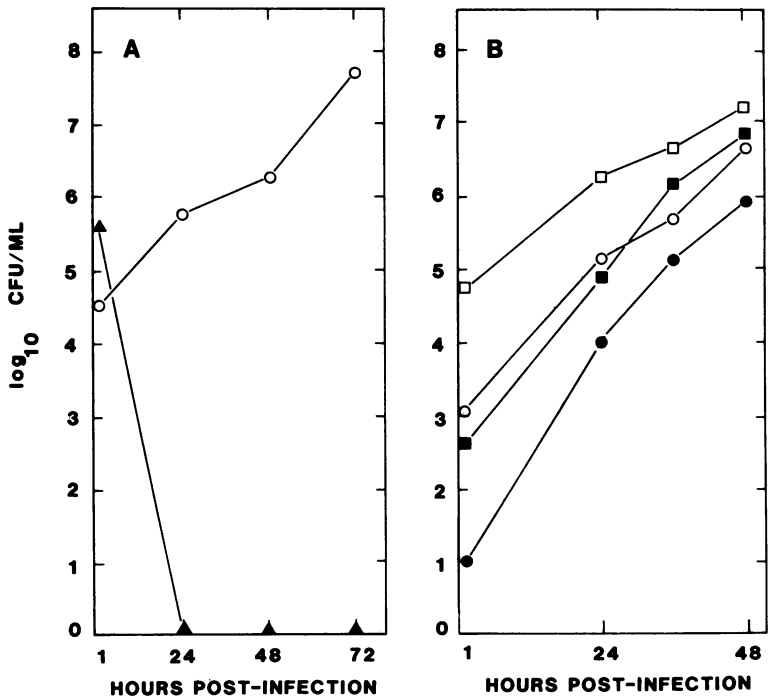

FIG. 1. Growth of *L. pneumophila* Philadelphia-1 in association with *A. castellanii* Neff. (A) Amoebae in AM were infected with *L. pneumophila* at a multiplicity of infection of 0.5 and an amoeba concentration of 4.3 × 10^5/ml. Samples were cultured on BCYE agar after disruption of amoebae by nitrogen cavitation (○). As a control, *L. pneumophila* was cultured in AM alone (▲). (B) Amoebae were infected as in (A) at multiplicities of infection of 1.0 (circles) and 2.3 (squares) and amoeba concentrations of 1.6 × 10^5/ml and 5.1 × 10^5/ml, respectively. Open symbols, Viable *L. pneumophila* released from infected amoebae by cavitation; closed symbols, viable *L. pneumophila* free in the medium of the infected culture. The three sets of curves were obtained in three separate experiments.

Amoeba-conditioned AM was prepared by incubating *A. castellanii* (ca. 10^5 amoebae per ml) in fresh AM for 72 h at 30°C. The culture medium was then filtered through a 0.45-μm membrane filter to remove amoebae and debris. The conditioned medium was used immediately without further treatment.

Acanthamoebae in amoeba-conditioned AM were added to a concentration of ca. 10^5 amoebae per ml to one compartment of one of the chambers. In this same chamber, ca. 10^4 to 10^5 legionellae per ml in amoeba-conditioned AM were loaded into the compartment opposite that containing the acanthamoebae alone.

In a control parabiotic chamber, one compartment was loaded with 10^5 amoebae which had been infected with legionellae and washed as described above. The opposite compartment of this chamber contained amoeba-conditioned medium alone.

Both parabiotic chambers were incubated at 30°C and sampled at 1, 24, 48, and 72 h after loading. At each time, a 10-ml sample was removed from the compartment in each chamber that did not contain amoebae. These samples were serially diluted in sterile distilled water and plated on BCYE agar medium. Samples (10 ml) were also removed from the compartment in both chambers that contained amoebae. These samples were processed by nitrogen cavitation-culture and stained by the Gimenez technique as described above.

Determination of intracellular location by differential fixation and fluorescent antibody analysis. Two cultures of *A. castellanii* were inoculated with *L. pneumophila* as described above, washed after 1 h of cocultivation, and incubated in fresh AM for 72 h post-inoculation. Samples were removed at 1, 24, 48, and 72 h post-inoculation and processed as described above. In addition to nitrogen cavitation and staining of the amoebae by the Gimenez technique, however, those amoebae resuspended in PAS were also processed by a differential fixation procedure. One group of cytocentrifuge slides was treated with cold acetone for 10 min at −5°C, washed in phosphate-buffered saline for 5 min, fixed with 1% Formalin at room temperature for 5 min, and rinsed in phosphate-buffered saline at room temperature for 5 min (12). The slides were then dried and immediately stained with type-specific fluorescent antibody as described by Cherry et al. (4).

The other group of slides was fixed with 2.5% glutaraldehyde at 6°C for 10 min and then washed in phosphate-buffered saline at room temperature for 10 min. These slides were also dried and immediately stained with type-specific fluorescent antibody.

Both types of slides were examined under epifluorescence illumination with a ×100 glycerol-immersion objective. The number of fluorescent rods associated with each of 200 amoebae was counted, and the percentage of infected amoebae and average number of legionellae per infected amoeba were calculated from these data as before.

Electron microscopy. A sample of amoebae cocultivated with virulent *L. pneumophila* for 48 h was removed from the culture and centrifuged at 200 × *g* for 10 min. The supernatant was removed and the pellet was suspended in 3 ml of PAS. The amoebae were then concentrated in an Eppendorf centrifuge, fixed for 24 h at 6°C with 2.5% glutaraldehyde (pH 7.3), and postfixed in 1% osmium tetroxide. The amoeba pellet was then dehydrated in a series of graded ethanol washes and embedded in Spurr epoxy resin. Sections were cut with an LKB Ultratome III X and then stained with uranyl acetate and lead citrate. All observations were performed with a Philips 301 transmission electron microscope.

RESULTS

Cocultivation of *L. pneumophila* and *A. castellanii* Neff. Cultures of *A. castellanii* were infected in suspension with *L. pneumophila* for 1 h, washed thoroughly to remove unassociated bacteria, and incubated at 30°C. When amoebae were removed from culture, centrifuged to remove free legionellae, disrupted by nitrogen cavitation, and cultured on BCYE agar, the level of viable *L. pneumophila* was shown to increase by more than three orders of magnitude over a 48-h period (Fig. 1A and B). The magnitude of the initial infection and the rate and extent of the growth of *L. pneumophila* were highly variable, with generation times varying from 4.4 and 8.8 h in these three representative experiments. These

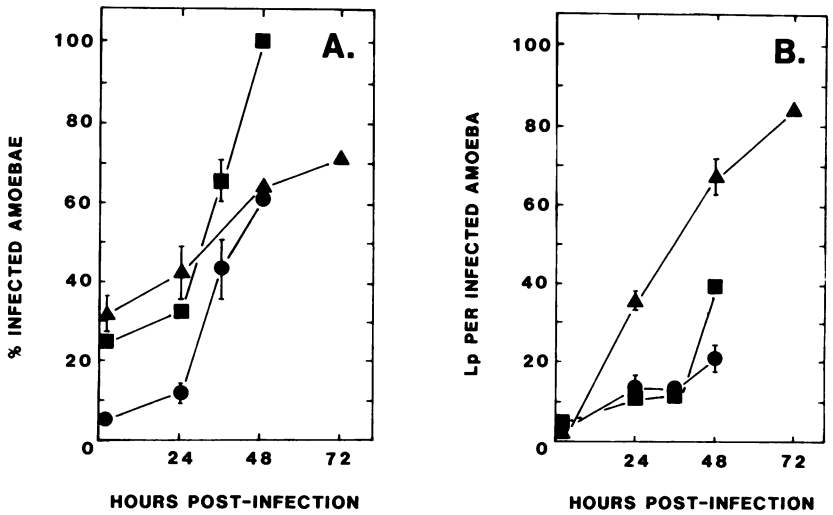

FIG. 2. Growth of *L. pneumophila* associated with *A. castellanii* as shown by staining by the Gimenez technique. Data are from the same cultures described in Fig. 1 at multiplicities of infection of 0.5 (▲), 1.0 (●), and 2.3 (■). (A) Percentage of amoebae infected with legionellae. (B) Average number of legionellae (Lp) per infected amoeba. Data points represent the mean ± standard deviation for legionella counts based on duplicate groups of 100 Gimenez-stained amoebae within each experiment.

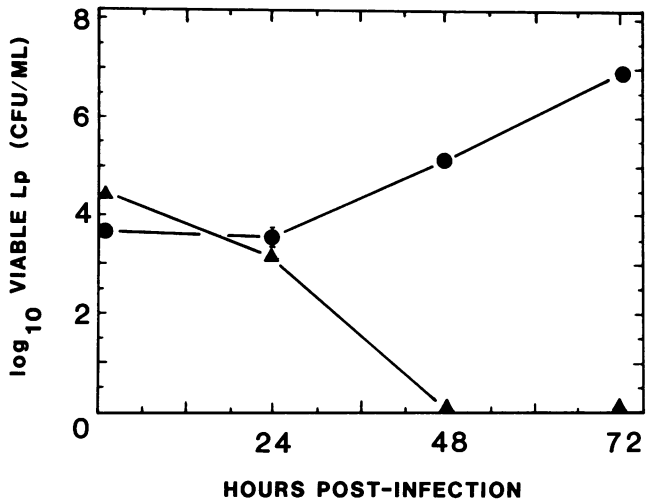

FIG. 3. Culture of *L. pneumophila* with *A. castellanii* in parabiotic chambers. Amoebae either were infected with *L. pneumophila* before culture in the parabiotic chamber (●) or they were separated from the legionella inoculum (in acanthamoeba-conditioned AM) by a 0.45-μm Nuclepore membrane (▲). Legionella counts from infected amoebae were obtained after disruption of washed amoebae by nitrogen cavitation.

parameters did not correlate well with the initial multiplicity of infection.

Although the number of viable legionellae free in the culture medium was initially much lower than the number associated with the amoebae, these levels of free legionellae also increased (Fig. 1B). They were, however, always considerably lower than the amoeba-associated counts. Control experiments demonstrated complete loss of viability in 48 h for *L. pneumophila* in AM alone, with >90% of this loss occurring within the first 24 h (Fig. 1A). These results suggested the possibility that *L. pneumophila* could also grow in medium conditioned by amoeba-derived soluble factors.

Gimenez staining of amoebae from infected cultures showed that both the percentage of legionella-infected amoebae and the number of legionellae per infected amoeba increased between each sampling period (Fig. 2). The increase in the percentage of infected amoebae during the first 24 h of cocultivation was generally only slight, with a rapid rise in infected amoebae between 24 and 48 h. Similarly, most of the increase in number of legionellae per infected amoeba occurred between 24 and 48 h post-inoculation. Many of the amoebae observed at the later time points contained large numbers of *L. pneumophila* which appeared to be densely packed within the cytoplasm. Because these amoebae were scored as containing 100 legionellae, the number of legionellae per infected amoeba is probably an underestimate late in infection. The number of total amoebae in the culture during the course of infection did not change appreciably in any of the experiments described in this report.

Multiplication of *L. pneumophila* in parabiotic chambers. Diffusion chambers consisting of two spinner flasks separated by a microporous membrane were used to investigate the possibility that a diffusible product also is involved in the growth of legionellae in cultures containing amoebae. When *L. pneumophila* were cultured in the same compartment with *A. castellanii*, disruption of the amoebae by nitrogen cavitation and culture of the amoeba lysate showed an increase in viable legionellae similar to that seen in cocultivation experiments (Fig. 3). In contrast, when *L. pneumophila* was added to the compartment which was separated from the amoebae by a membrane filter, the legionellae did not grow and in fact did not survive more than 48 h in the culture medium even though this medium had been preconditioned by incubation for 72 h with amoebae.

Sampling of compartments to which no legionellae were added demonstrated that the bacteria did not cross the membrane filter. In addition, control experiments in which trypan blue was included in the chamber containing the amoebae indicated that free diffusion of dye through the membrane filter was not hindered by the presence of the

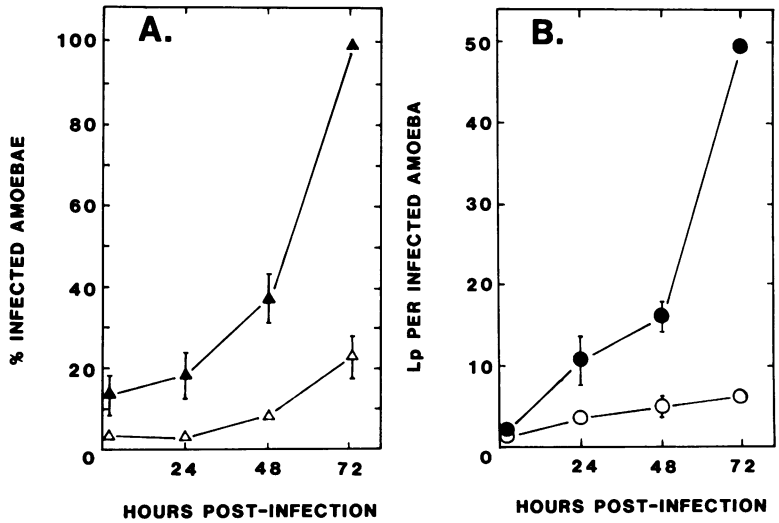

FIG. 4. Intracellular growth of *L. pneumophila* in *A. castellanii* as shown by differential fixation and direct fluorescent antibody. Results show the change in percentage of infected amoebae (A) and in average number of legionellae (Lp) per infected amoeba (B). Amoebae either were permeabilized to the fluorescent-labeled anti-legionella antibody by cold acetone-Formalin fixation (solid symbols) or were fixed with glutaraldehyde to prevent permeation of the labeled antibody (open symbols). Data points represent the mean ± standard deviation of results from two separate experiments with duplicate infections in each experiment. Where no error bars are visible, the magnitude of the standard deviation was less than the size of the symbol.

amoebae. Thus, *L. pneumophila* underwent multiplication only when direct contact with amoebae was possible.

Intracellular localization of *L. pneumophila* by differential fixation. Gimenez staining of amoebae cocultivated with legionellae does not distinguish between adherent and intracellular bacteria. To demonstrate that the number of intracellular bacteria increases concomitantly with the increase in legionellae as demonstrated by cavitation and culture, we combined a differential fixation method with direct fluorescent antibody analysis. Amoebae were treated with cold acetone before fixation with Formalin when permeation of fluorescent anti-legionella antiserum was desired and with 2.5% glutaraldehyde when only extracellular labeling was desired (12).

Samples of amoebae treated with cold acetone before fixation and staining showed a steady increase in the number of legionellae per infected amoeba over a 72-h period (Fig. 4). In contrast, there was little change in the number of legionellae per infected amoeba when amoebae from the same culture were fixed with 2.5% glutaraldehyde and then stained with fluorescent antibody. Because the fluorescent antibody stains primarily extracellular bacteria when amoebae are fixed with glutaraldehyde, the counts performed on these cells were interpreted to represent the number of adherent bacteria in the sample. The percentage of infected amoebae increased in the sample treated with cold acetone as it did in samples stained by the Gimenez technique; almost 100% of the amoebae contained fluoresent bacteria by 72 h post-inoculation. The percentage of infected amoebae in the glutaraldehyde-fixed sample did not change for the first 24 h and increased only slightly by 72 h when compared with counts taken from amoebae fixed with cold acetone. Increases in the percentage of infected amoebae in the glutaraldehyde-fixed samples were probably due at least in

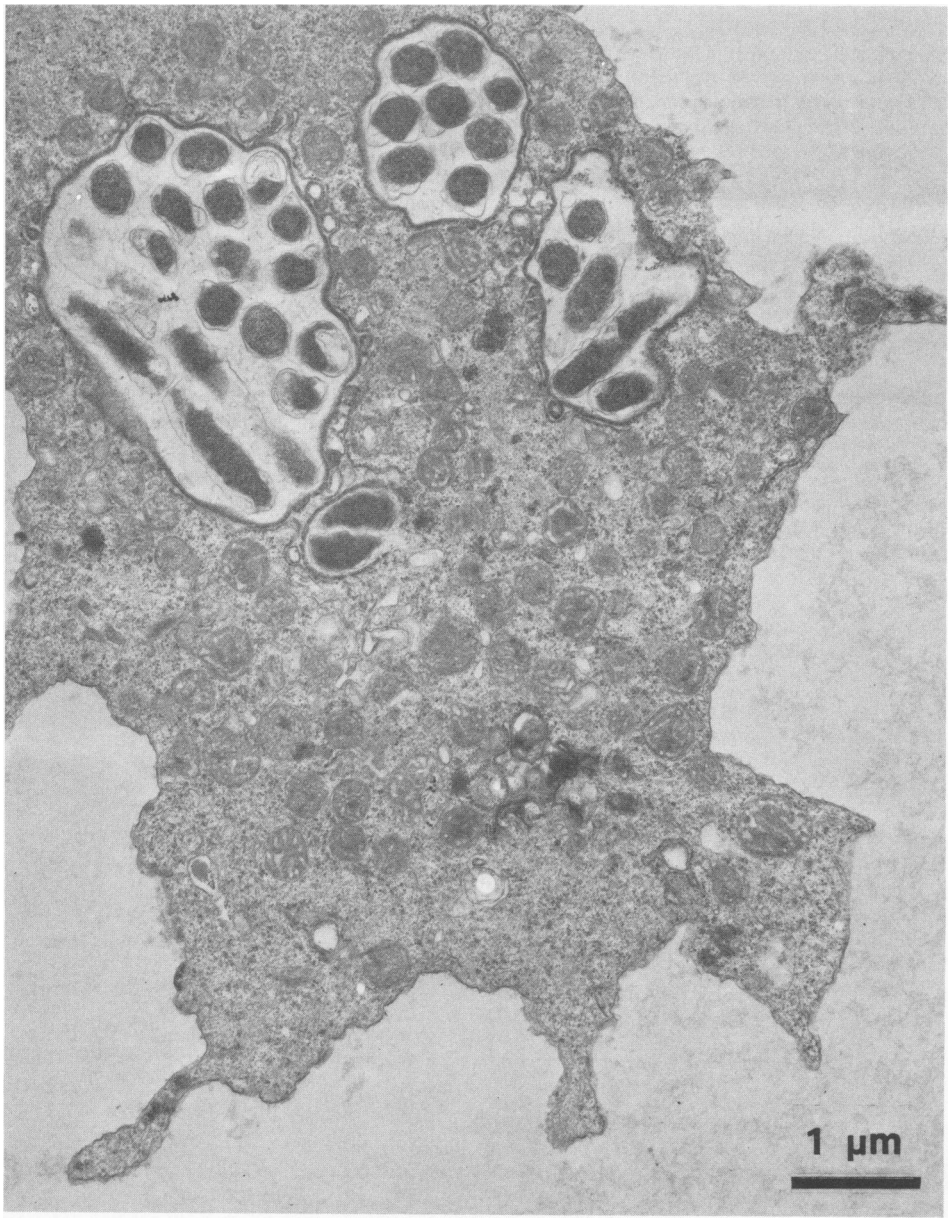

FIG. 5. Transmission electron micrograph of *L. pneumophila* within *A. castellanii* at 48 h postinfection. Note the intravacuolar locus of bacteria and the presence of apparent dividing forms. Magnification, ×21,300.

part to the apparent fragility of heavily infected amoebae, leading to an increase in the permeability of the plasma membrane to fluorescent antibody.

Electron microscopy. Amoebae infected with *L. pneumophila* were fixed with glutaraldehyde and examined by transmission electron microscopy. Numerous bacteria were observed in random ultra-thin sections of amoebae when viewed under the transmission electron microscope (Fig. 5). *L. pneumophila* were observed to reside within membrane-bound cytoplasmic vacuoles in samples prepared 48 h after infection. The number of bacteria within each vacuole ranged from a few in lightly infected cells to over 100 in some of the more heavily infected cells. Dividing bacteria could also be observed within the vacuoles in a number of the amoebae examined. Observations of the surface of the amoebae with the scanning electron microscope showed only an occasional bacterium-like structure attached to the cell surface of an amoeba (data not shown).

DISCUSSION

L. pneumophila grew rapidly when cocultured with the Neff strain of *A. castellanii*, although the culture medium by itself supported neither the growth nor the survival of legionellae. The mean generation time for legionellae growing in association with amoebae was 5.2 h (± 2 h) based on viable counts from amoeba lysates in the experiments presented in Fig. 1, 3, and 4. Under the conditions of these experiments, only a low number of legionellae were phagocytized during the infection period; however, the total number of viable legionellae in the system, the percentage of amoebae infected with legionella, and the mean number of bacteria per infected amoeba increased rapidly over the next 72-h period. The magnitude of each of these parameters sometimes varied widely between experiments, most likely due to differences in the physiological state of both the amoebae and the legionellae.

Nonetheless, in most of the experiments to date, the pattern of this increase is consistent with intracellular, or at least amoeba-associated, growth followed by rupture of the infected amoebae and release of legionellae for initiation of another round of infection. It is also possible, however, that the increasing levels of viable legionellae found free in the culture medium during infection are attributable at least in part to release of membrane-bound "packets" of legionellae such as the small vescicles described by Rowbotham (18).

These results support the conclusions of Rowbotham (17) and of Tyndall and Domingue (22) that members of the genera *Acanthamoeba* and *Naegleria* provide either an intracellular niche or extracellular factors for the growth of *L. pneumophila*.

If diffusible extracellular factors contribute to legionella growth, then growth should occur when the legionellae are physically separated from the amoebae by a barrier which permits diffusion of soluble factors but prevents amoeba-bacterium contact. When growth of *L. pneumophila* and *A. castellanii* was studied with parabiotic chambers, however, legionella growth occurred only when contact with the amoebae was possible; otherwise, the levels of viable legionellae declined as in culture medium alone. These results are analogous to those obtained by Horwitz and Silverstein (11) for the interaction between human monocytes and *L. pneumophila* and differ from the effect of cyanobacteria on legionella growth (D. L. Tison, D. H. Pope, and C. B. Fliermans, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I40, p. 91). Thus, if extracellular factors are involved in legionella growth they are either very short-lived or they remain amoeba associated.

Intracellular growth of the legionellae was supported by both immunofluorescence and transmission electron microscopy data. Amoebae fixed so as to permeabilize them to fluorescent anti-*L. pneumophila* antibody showed the same rapid increase in the percentage of infected amoebae and mean legionellae per infected amoeba seen with Gimenez stain. In contrast, amoebae fixed under conditions which did not render them permeable to antibody showed very little change in the (low) level of associated legionellae over a 72-h period. The appearance of large numbers of bacteria, some of them apparently in the process of division, within cytoplasmic vacuoles in electron micrographs of *A. castellanii* 48 h after infection is evidence for an intracellular locus for the growth of *L. pneumophila*.

The implications of the intra-amoebal growth of *L. pneumophila* for the environmental biology of legionellae are significant. Not only are acanthamoebae able to promote legionella growth as are the blue-green algae (16), but the intracellular locus of this growth may well contribute to the difficulty encountered in removing legionellae from cooling towers and similar environments by routine maintenance procedures (7). As shown by Skinner et al. (19), legionellae can be observed within acanthamoeba cysts under experimental conditions. If *L. pneumophila* can survive the differentiation of acanthamoebae into the cyst form, intracellular residence could provide even greater protection for longer periods due to the unusual stability of such cysts to such treatments as hyperchlorination (5). In addition, infected amoebae could provide a reservoir for legionella survival when conditions such as temperature and light intensity become unfavorable for growth of other supporting organisms such as cyanobacteria.

Tyndall and Domingue (22) were unable to demonstrate an alteration of either legionella or amoeba virulence associated with this parasitism. This observation does not, however, preclude a role for amoebae in maintaining legionella virulence in the environment. In preliminary experiments we noted a difference in the interaction between *A. castellanii* and low-passage versus high-passage strains of *L. pneumophila* on solid agar media. Whether in fact this difference will reflect an actual ability of *A. castellanii* to discriminate between virulent and avirulent strains remains to be seen. Comparison of the uptake and growth kinetics of strains of defined virulence for an animal model will require culture conditions which allow better reproducibility in the physiological state of the amoeba and legionella inocula.

The results of this study clearly demonstrate the existence of an intracellular niche for legionella growth, similar to that already demonstrated in human monocytes (11). Of central importance now is an understanding of the cell biology of this unusual interaction and a demonstration of the natural occurrence of this phenomenon in aquatic habitats of the type implicated in legionellosis.

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