Survival of *Legionella pneumophila* in the Cold-Water Ciliate *Tetrahymena vorax*

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Received 8 April 1991/Accepted 10 July 1991

The processing of phagosomes containing *Legionella pneumophila* and *Escherichia coli* were compared in *Tetrahymena vorax*, a hymenostome ciliated protozoan that prefers lower temperatures. *L. pneumophila* did not multiply in the ciliate when incubated at 20 to 22°C, but vacuoles containing *L. pneumophila* were retained in the cells for a substantially longer time than vacuoles with *E. coli*. Electron micrographs showed no evidence of degradation of *L. pneumophila* cells through 12 h, while *E. coli* cells in the process of being digested were observed in vacuoles 75 min after the addition of the bacterium. *T. vorax* ingested *L. pneumophila* normally, but by 10 to 15 min, the vacuolar membrane appeared denser than that surrounding nascent or newly formed phagosomes. In older vacuoles, electron-dense particles lined portions of the membrane. Acidification of the phagosomes indicated by the accumulation of neutral red was similar in *T. vorax* containing *L. pneumophila* or *E. coli*. This ciliate could provide a model for the analysis of virulence-associated intracellular events independent of the replication of *L. pneumophila*.

*Legionella pneumophila*, a facultative intracellular pathogen with a permissible temperature range for growth of 25 to 42°C (32), has been isolated from aquatic environments including conventional cooling towers, streams, lakes, and potable water sources (6, 7, 11, 16, 21, 23). The ability of *L. pneumophila* to survive and reproduce in freshwater amoebae and *Tetrahymena* spp. (2, 3, 9, 10, 25, 31) has been suggested to provide protection for the bacterium from lower temperatures during the winter months (3). This relationship potentially could permit the existence and propagation of *L. pneumophila* in chilled water aquifer thermal energy storage systems in which aquifer water is intimately exposed to air by spray to chill the water from 17 to 22°C or less for aquifer storage. Currently, only one aquifer thermal energy storage system is operating in the United States, and this prototype system has demonstrated the potential for notable energy savings in temperate regions (18). Previous investigators have examined the survival of *L. pneumophila* within protozoa at temperatures within the permissible range for growth of both bacteria and protozoa, including *Tetrahymena pyriformis*. To investigate the possible survival of *L. pneumophila* with protozoa active at lower temperatures, we selected *T. vorax* as a model host. This species has an optimum growth temperature of 20°C. Temperatures of 30°C are lethal.

*T. vorax* is a hymenostome ciliate which in nature feeds by filtration and concentration of bacteria and other particulate material into food vacuoles (phagosomes) at the base of the cytopharynx of the oral apparatus. After separation from the cytopharynx, the phagosome enters a processing period during which the vacuole decreases in size and undergoes an initial acidification stage. This is followed by a digestive stage in which the vacuole contents are hydrolyzed by enzymes acquired by fusion with lysosomes. The final stage in the digestive cycle is the fusion of the vacuole with the cytoproct, a defined region of the cell surface, for release of undigested material. In *T. vorax*, the timing of the stages of the digestive cycle has been determined by vacuole pH, acid phosphatase activity of isolated vacuoles, and initiation of the defecation period for vacuoles labeled with ferric oxide (29). Acidification begins shortly after detachment of the phagosome from the cytopharynx and is essentially complete by 30 min, whereas the digestive phase initiated by the fusion of lysosomes is entered between 30 and 60 min. The end of the processing period and the beginning of the defecation period occurs at 100 min. In this report, we compare the processing of vacuoles containing *L. pneumophila* with that of vacuoles with *Escherichia coli*, a bacterium normally digested by *Tetrahymena* spp.

**MATERIALS AND METHODS**

**Cultures.** *L. pneumophila* serogroup 1 (Philadelphia 1 strain) was obtained from James Barbaree (Centers for Disease Control, Atlanta, Ga.). Cryogenic cultures were inoculated onto buffered charcoal-yeast extract agar containing L-cysteine (0.4 mg/liter) and incubated at 35°C under humidified air and 2.5% CO₂ for 4 to 7 days. Bacteria were harvested by flooding the plate with sterile tap water, and the cell density was adjusted to a McFarland no. 5 standard. Samples were fixed in 2.5% (vol/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in modified inorganic medium (27), and the optical density (A₅₅₀) was measured. Dilutions of 10⁻⁶, 10⁻⁷, and 10⁻⁸ were plated in duplicate, and the cell density was determined as CFU.

*E. coli* Y1090 (Promega, Madison, Wis.) was inoculated into LB broth and incubated overnight. The bacteria were concentrated by centrifugation and suspended in distilled water. The optical density (A₅₅₀) was adjusted to that of the *L. pneumophila* suspensions, or the cell density was adjusted to a McFarland no. 5 standard.

**Cultures of *T. vorax*** (Kidder 1941) strain V₄S were started by transferring 10 ml of a stationary-phase culture into 50 ml of sterile Loefler’s medium (17) contained in a 500-ml Erlen-
meyer flask. The protozoa were grown for 2 days at 20°C. Cell density was determined with a Coulter Counter.

Retention and growth of bacteria. Bacteria were stained for 10 min with 1 or 10 μg of 4',6-diamidino-2-phenylindole (DAPI) per ml, depending on the intensity of the fluorescence obtained with each preparation of the dye. The cells were pelleted, washed in inorganic medium, and suspended in inorganic medium to the initial volume. Bacterial plate counts were performed to confirm viability. In some experiments, T. vorax was washed three times in inorganic medium, suspended to the initial volume, and incubated for 1 h prior to the addition of the bacteria. The bacterial and T. vorax suspensions were combined at a bacteria/protozoa ratio of ~10⁵/¹ and incubated at room temperature (20 to 22°C) for 5 min. Initially, different ratios of T. vorax and bacteria were mixed to observe the ingestion of bacteria during a 5-min pulse. The 10⁵/¹ ratio of bacteria to protozoa was necessary to produce a consistent number of protozoa containing vacuoles with bacteria. Lower bacterium/protozoa ratios yielded large numbers of protozoa with no vacuoles containing bacteria and a highly variable number of bacteria per vacuole in those protozoa with labeled vacuoles. At the end of the pulse, the suspension was centrifuged for 10 s at top speed in an IEC clinical model centrifuge. The pellet was washed three times in inorganic medium, suspended in inorganic medium to the initial volume of the protozoan suspension, transferred to a 50-ml Erlenmeyer flask, and maintained at room temperature for the chase period of the experiment. For longer experiments, the pellet was suspended in Loefer’s medium, and 2.5 ml were placed in 10 ml of Loefer’s medium in a 125-ml Erlenmeyer flask. Samples were removed at intervals designated by the elapsed time following the addition of the bacteria and placed in an equal volume of 5% (vol/vol) glutaraldehyde and 4% (wt/vol) paraformaldehyde in inorganic medium. The number of vacuoles containing DAPI-stained bacteria was counted by using a Leitz Ortholux II microscope equipped with epifluorescence optics, and the mean number of vacuoles per 100 cells at each time was determined. For protozoa suspended in fresh Loefer’s medium for the chase period, the mean was normalized to the initial density of the protozoa to adjust for cell division. Protozoa were photographed with Kodak T-Max 400 or Ektar 125 film by using a Nikon Optiphot microscope equipped with epifluorescence optics and a Microflex UFX-II photographic attachment.

To estimate the number of CFU of L. pneumophila at each time, the procedure was repeated with unstained bacteria. Samples were removed at designated intervals, and dilutions of 10⁻³, 10⁻⁶, and 10⁻⁹ were plated.

Electron microscopy. T. vorax and unstained L. pneumophila, E. coli, or a suspension of equal volumes of both bacteria were combined and processed as described above. Aliquots were removed at the specified intervals and placed in an equal volume of either 5% (vol/vol) glutaraldehyde or 5% (vol/vol) glutaraldehyde and 4% (wt/vol) paraformaldehyde in inorganic medium. Samples were fixed in the primary fixative for 20 min, washed three times in inorganic medium, and postfixed in 1% osmium tetroxide in inorganic medium for 30 min. Following dehydration in a graded ethanol series, the cells were embedded in Spurr’s low-viscosity resin (30). Sections were cut with a Sorvall MT 5000 microtome, stained with uranyl acetate and lead citrate, and viewed with a Zeiss EM10A electron microscope operated at 60 kV.

Acidification of vacuoles. T. vorax was washed and suspended in inorganic medium, pH 7.0, and incubated for 1 h. The pH of the medium was checked after the incubation period. The protozoa were pulsed with DAPI-stained bacteria for 5 min and chased in inorganic medium. At intervals during the chase period, the protozoa were stained with neutral red (50 μg/ml) for 1.5 min, rinsed with inorganic medium to remove the free base, and fixed in 1% (vol/vol) glutaraldehyde in inorganic medium. The samples were viewed and photographed immediately. A red color of a vacuole containing bacteria indicated that the vacuole was acidic. Neutral red diffuses rapidly across the membranes in the free-base form but becomes trapped in vesicles with acidic pH because of the reduced permeability of the protonated form (24). This technique permits qualitative identification of acidic compartments which appear red as a result of the accumulation of the protonated dye.

RESULTS

When either bacterium was supplied to T. vorax as its only food source, the mean number of vacuoles per cell containing fluorescent L. pneumophila remained essentially constant over a 6-h period, while the number of vacuoles containing the control bacterium E. coli began to decline between 60 and 70 min (Fig. 1), well before the beginning of the defecation period at 100 min (29). L. pneumophila also persisted in T. vorax supplemented with Loefer’s medium to eliminate starvation effects, but the mean number of vacuoles with fluorescence characteristic of that after the 5-min pulse with L. pneumophila decreased gradually but significantly (P < 0.001) over 12 h (Fig. 2). Initially, few bacteria were present free in the medium, but after 2 to 3 h of the chase period, fluorescent spheres resembling vacuole contents were observed in the medium, and some ciliates with vacuoles containing only one to a few L. pneumophila cells in addition to the vacuoles formed during the 5-min pulse were noted. Some protozoa had individual bacteria throughout the cell; these protozoa often appeared to be dead or dying. By 24 h, few ciliates contained discrete heavily labeled vacuoles, but the number of viable cells of L. pneumophila (~10⁵ CFU) remained constant over this time. L. pneumophila remained viable but did not grow on buffered charcoal-yeast extract agar medium at 20 to 22°C or
in Loeffler's medium either at 20 to 22°C or at 35°C. The bacterium also did not multiply in T. vorax during the 12-h period at 20 to 22°C, nor did the number of CFU increase when the time was extended to 48 h (data not shown). T. vorax underwent cell division when resuspended in fresh Loeffler's medium, but the increase in cell density was less for populations that had ingested L. pneumophila relative to controls without the bacterium (Fig. 3).

Figures 4 and 5 illustrate vacuoles containing L. pneumophila and E. coli at different times after the addition of bacteria. These bacteria differ in appearance in electron micrographs, with L. pneumophila observed as substantially smaller than E. coli and less coccobacillary in shape. During ingestion, L. pneumophila was located in vacuoles formed at the cytopharynx of T. vorax (Fig. 4A). When long bacteria were ingested, they often caused the shape of the vacuole to deviate from the normal spherical shape (Fig. 4B). Electron-lucent vesicles occurred near the membrane of phagosomes before and immediately after separation from the cytopharynx, while the vacuole is associated with the deep fiber (Fig. 4C). In some sections, these vesicles appeared to be fusing with the vacuolar membrane. Initially, the bacteria were dispersed within the vacuole (Fig. 4A through C and 5A), but shortly after vacuole formation, both L. pneumophila and E. coli were compressed and close to the phagosomal membrane (Fig. 4B, 4D, and 5B). While cells of E. coli were often in contact with each other and appressed to the membrane (Fig. 5B), those of L. pneumophila were surrounded by an electron-lucent zone (Fig. 4D). By 75 min, lysosomes were adjacent to the membrane of some vacuoles containing E. coli, and many of the bacteria no longer showed the finely granular appearance of intact cells (Fig. 5C). The contents of these vacuoles frequently were separated from the membrane by a space. At 120 min, the vacuoles ranged from those with bacteria similar in appearance to bacteria in vacuoles at 75 min to vacuoles containing remnants of cell walls with few to no intact bacteria (Fig. 5D through F). Vacuole fusion was also observed (Fig. 5F). In contrast, L. pneumophila showed no signs of degradation through 12 h (Fig. 4E). The vacuoles were irregular in shape, with bacteria in invaginations. When L. pneumophila and E. coli were ingested together, both types of bacteria appeared to be digested during a processing time similar to that for E. coli alone (Fig. 4F). No vacuoles containing bacteria were observed in 6-h samples.

The membrane of phagosomes in protozoa fixed immediately after a 5-min incubation with L. pneumophila was similar to that delimiting vacuoles in control cells containing E. coli throughout the digestive cycle (Fig. 4A through C, 5, and 6A); however, in samples washed and suspended before fixation (10 to 15 min after addition of the bacteria) and those at 30 min, most vacuoles appeared to be lined by a dense membrane (Fig. 4D and 6B). A few mitochondria were observed appressed to the membrane from this time through 12 h (Fig. 4D and E). Electron-dense particles similar to cytoplasmic ribosomes were present near the vacuole, but it is unclear whether these are aligned along the membrane in a manner characteristic of older vacuoles (Fig. 6C) until after 30 min. At later times, the membrane also lacked the density. Unlike the dense membrane which appears to border the entire surface of younger vacuoles, the electron-dense particles do not completely line the membrane but are almost always present along the membrane adjacent to

FIG. 4. Electron micrographs of sections through vacuoles in T. vorax containing L. pneumophila. Bar, 1 μm. (A) Nascent vacuole in cell incubated with L. pneumophila for 5 min before fixation. (B) Section through two vacuoles formed during a 5-min incubation with bacteria before fixation. Note the shape of the vacuole containing a long bacterium. (C) Section through vacuole formed during a 5-min pulse with L. pneumophila. Note the electron-lucent vesicles around the phagosome (arrow) and the deep fiber (arrowhead), indicating that this is a newly formed vacuole near the oral apparatus. Inset shows a section through an electron-lucent vesicle fusing with a vacuole (bar, 0.1 μm). (D) Section through a 30-min-old vacuole. Note clear spaces around bacteria, the dense appearance of the vacuole surface and the mitochondrion appressed to the membrane (arrow). (E) Section through a 12-h-old vacuole. Note presence of bacteria in invaginations (arrows) and the granular appearance along phagosomal membrane (arrowhead). Two mitochondria are appressed to the membrane (double arrows). (F) Section through 120-min-old vacuole containing both E. coli (large cells, arrow) and L. pneumophila (small cells, arrowhead). Both bacteria appear to be undergoing degradation.
bacteria. Vacuoles in control protozoa incubated with *E. coli* showed no evidence of similar electron-dense particles around the membrane at any stage during the digestive cycle (Fig. 6A).

Acidification was similar for vacuoles with *L. pneumophila* and *E. coli*. At 30 min after the introduction of the bacteria, there was no detectable difference in the accumulation of neutral red by vacuoles containing either *E. coli* (Fig. 7A and B) or *L. pneumophila* (Fig. 7C and D). Vacuoles with *L. pneumophila* gave comparable results when chased with inorganic medium up to 90 min.

**DISCUSSION**

The results of this study show that while *L. pneumophila* does not multiply in *T. vorax* at temperatures below its minimum growth temperature of 25°C (32), it is retained in phagosomes substantially longer than the normal processing period. While fluorescence of labeled *E. coli* began to disappear from vacuoles during the digestive stage and electron micrographs showed degradation of the bacterium, vacuoles containing fluorescent *L. pneumophila* did not decrease when the protozoa were maintained in stationary phase for 6 h. The mean number of fluorescent phagosomes did decline when *T. vorax* was returned to logarithmic growth by resuspending the cells in fresh Loefer’s medium. This decrease does not appear to be due to digestion of the bacterium, since no evidence of degradation was seen in electron micrographs through 12 h and the number of viable *L. pneumophila* present in these samples remained constant. The decline may be due to defecation of the vacuoles without digestion of the contents in logarithmically growing cells. Alternatively, these protozoa may be more sensitive to virulent *L. pneumophila* than protozoa in stationary phase, which could result in the death of some protozoa, particularly those with a greater number of vacuoles containing bacteria. This could account not only for the continuous

![Image of sections through vacuoles](image-url)
decline in the mean number of labeled vacuoles for T. vorax in logarithmic growth while that for protozoa in stationary phase remained constant, but also for the smaller increase in cell density of the protozoa in the presence of L. pneumophila than in control populations of protozoa without the bacterium, the presence of spheres with fluorescent bacteria in the medium (which may represent vacuole contents released upon cell death), and the inclusion of low numbers of bacteria in phagosomes after incubation in Loefer’s medium for several hours possibly from ingestion of bacteria released into the medium. Weidenbach and Thompson (33) have reported that vacuoles are more fragile and difficult to isolate from Tetrahymena thermophila in logarithmic phase than from protozoa in stationary phase. It is interesting to note that a surprising number of macrostomial cells, a cell type into which T. vorax usually differentiates when starved and provided with an additional stimulus (4, 5, 15), were observed in samples removed during the later times of incubation (28), although the Loefer’s medium should provide sufficient nutrients for growth throughout the period.

The failure of T. vorax to digest L. pneumophila at a temperature unfavorable to multiplication of the bacterium differs from the results of Anand et al. (2) for Acanthamoeba palestinensis. They reported that although L. pneumophila survives and reproduces in vacuoles at 35°C, this protozoan digests the bacterium at 20°C. This may be due to the different concentrations of L. pneumophila present in the vacuoles of the two protozoa. Only one to several bacterial cells were contained in vacuoles of this protozoan, while T. vorax ingested a large number of L. pneumophila cells when the bacterium was provided to the protozoa at the cell density used in this study. When E. coli and L. pneumophila were ingested together, fewer cells of L. pneumophila were included in each vacuole, and the bacterium appeared to be degraded in a manner similar to that of E. coli. It is conceivable that decreased metabolism in L. pneumophila at the lower temperature necessitates the presence of a greater number of bacteria to block digestion. Studies on the interaction of L. pneumophila with human monocytes have indicated that viable, metabolically active bacteria are required to inhibit the phagocytic pathway (12-14, 19). Alternatively, E. coli or products of this bacterium could counteract the inhibition by L. pneumophila by interacting either with cells or products of L. pneumophila or with the membrane of the vacuole.

Unlike human monocytes and amoebae which undergo phagocytosis by surrounding the particle with membrane derived from the plasmalemma, Tetrahymena spp. form vacuoles by the addition of membrane to the base of the cytopharynx (20, 26). Vacuole formation and ingestion of L. pneumophila occurred normally. Electron-lucent vesicles were adjacent to the phagosome before and immediately after separation from the cytopharynx, and in several instances they appeared to be fusing with the vacuole. These vesicles resemble the acidosomes of Paramaecium spp., which are believed to be responsible for acidification of the phagosome (1) but which have not been observed in Tetrahymena spp. previously. Acidification of vacuoles indicated by the accumulation of neutral red was similar for vacuoles containing L. pneumophila and E. coli; however, this method only indicates acidic compartments within the cell and does not measure the actual pH. Horwitz and Maxfield (14) found that the pH of monocyte vacuoles with live L. pneumophila was ~6.1, averaging 0.8 pH units higher than that for dead cells and 0.4 pH units above that for vacuoles containing E. coli. This pH is well above the minimum pH of 5.5 for multiplication of L. pneumophila (32). In a previous study, we found that the pH of vacuoles in T. vorax dropped to ~6 within 10 min after formation and reached a minimum pH of 4.9 by 30 min (29). It is possible that although the vacuole with L. pneumophila begins the process of acidification, a change in the membrane or underlying region of the cytoplasm at around 10 to 15 min inhibits a further decrease in pH. Such a change could be induced by a product or products of L. pneumophila and could result in the difference in the appearance of the vacuole surface after this time. This alteration in the membrane or the presence of underlying electron-dense particles could block the fusion of lysosomes, as has been reported in monocytes (13). The modification in the membrane and underlying region of the vacuole precedes the time of lysosomal fusion, as determined by measurement of acid phosphatase activity in vacuoles of T. vorax isolated at different times in the processing period (29).

Similar observations on the membrane of vacuoles containing L. pneumophila undergoing multiplication have been reported in T. pyriformis (8), Naegleria fowleri (22), and human monocytes (12). In macrocytes, vacuoles with L. pneumophila often had one or more mitochondria apposed to the vacuolar membrane, a situation also observed in T. vorax; however, those in monocytes were also surrounded by smooth vesicles in the process of either fusing with or budding off from the vacuolar membrane to at least 4 h after infection. Similar vesicles were not present around the vacuoles in T. vorax.

The behavior of L. pneumophila in the cold-water species T. vorax in the absence of bacterial growth is comparable to that described for other cells under conditions favorable for bacterial growth. This ciliate could provide a model in which to analyze the sequence of virulence-associated intracellular events independent of the replication of L. pneumophila.

ACKNOWLEDGMENTS

We thank Catherine Shea for her helpful suggestions and her assistance with preparation of the graphs.

This work was supported in part by the School of Mines and Energy Development, University of Alabama, and the U.S. Department of Energy through Pacific Northwest Laboratory via subcontract 005383-A-O. The Pacific Northwest Laboratory is operated for the U.S. Department of Energy by Battelle Memorial Institute.

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