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A Quantitative Model of Intracellular Growth of *Legionella pneumophila* in *Acanthamoeba castellanii*

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A model of intracellular growth for *Legionella pneumophila* in *Acanthamoeba castellanii* has been developed and provides a quantitative measure of survival and replication after entry. In this model, *Acanthamoeba* monolayers were incubated with bacteria in tissue culture plates under nutrient-limiting conditions. Gentamicin was used to kill extracellular bacteria following the period of incubation, and the number of intracellular bacteria was determined following lysis of amebae. Intracellular growth of virulent *L. pneumophila* and other wild-type *Legionella* species was observed when the assay was performed at 37°C. At room temperature, none of the *Legionella* strains tested grew intracellularly, while an avirulent *L. pneumophila* strain was unable to replicate in this assay at either temperature. The effect of nutrient limitation on *A. castellanii* during the assay prevented multiplication of the amebae and increased the level of infection by *Legionella* spp. The level of infection of the amebae was directly proportional to the multiplicity of infection with bacteria; at an inoculum of 1.03×10^7 bacteria added to wells containing 1.10×10^5 amebae (multiplicity of infection of 100), approximately 4.4% of *A. castellanii* cells became infected. Cytochalasin D reduced the uptake of bacteria by the amebae primarily by causing amebae to lift off the culture dish, reducing the number of target hosts; methylamine also reduced the level of initial infection, yet neither inhibitor was able to prevent intracellular replication of *Legionella* spp. Consequently, once the bacteria entered the cell, only lowered temperature could restrict replication. This model of intracellular growth provides a one-step growth curve and should be useful to study the molecular basis of the host-parasite interaction.

Legionella pneumophila, a cause of severe and often fatal pneumonia in humans, is a member of a large genus of slender, gram-negative rods that are facultative intracellular parasites. In humans and experimentally infected guinea pigs, *L. pneumophila* cells enter and replicate within alveolar macrophages (15), and in the human macrophagelike cell line U-937, uptake, survival, and replication of *L. pneumophila* resemble the steps in infection of macrophages (17). In nature, legionellae are found in fresh water and soil as parasites of protozoa, and in vitro, *Tetrahymena* (9), *Naegleria* (18, 21), *Acanthamoeba* (1, 11, 18, 21), and *Hartmanella* (14, 22) species have been shown to support the intracellular growth of *L. pneumophila* and other *Legionella* species (9). These species, especially hartmannellid amebae (22), have been isolated from water sources containing *Legionella* spp. during outbreaks of disease (2), suggesting that protozoans serve as important reservoirs in nature of human infection.

To further examine the host-parasite relationship and to identify bacterial factors that permit growth of *Legionella* spp. in protozoan hosts, we have developed a quantitative model of intracellular bacterial replication, using the small, free-living ameba *Acanthamoeba castellanii*. This model differs from the protozoan models used by others (1, 9, 11, 22) in which the amebae are permitted to replicate simultaneously with intracellular bacterial replication, making it difficult to dissect the steps which occur during the first round of growth following entry. Since under these circumstances it might be more difficult to discern the effects of inhibitors of phagocytosis and endocytosis or to monitor protein synthesis, we developed a new model which could more easily permit us to examine different phases in intracellular growth. This model was patterned after an assay to measure invasion of members of the family *Enterobacteriaceae* into epithelial cell lines (10) in which gentamicin is utilized to kill extracellular bacteria. Therefore, the number of CFU measured depends on the ability of legionellae to avoid extracellular killing by entry into the cell followed by intracellular replication. Using this model, we compared the ability of virulent and avirulent *L. pneumophila* and other *Legionella* species to enter and to replicate in *A. castellanii* and in U-937 cells, a transformed human monocytic cell line, under different conditions. The effects of assay temperature, nutrient limitation, and multiplicity of infection (MOI) were examined in order to characterize the model and to provide the optimal conditions for intracellular bacterial growth.

MATERIALS AND METHODS

Culture conditions. Axenic *A. castellanii* ATCC 30234 (American Type Culture Collection, Rockville, Md.) was raised at room temperature (23 to 25°C) in 15-ml screw-cap tubes (Falcon 2025; Becton Dickinson and Co., Lincoln Park, N.J.) in 6 ml of PYG broth [2% proteose peptone no. 3, 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), 0.1 M glucose, 4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3 (Sigma Chemical Co., St. Louis, Mo.), pH 6.5]. Amebae were grown to confluence on the tube by 48 h. Bacterial strains were maintained as freezer stocks in 25% glycerol at −70°C and passaged no more than three times on buffered charcoal-yeast extract agar supplemented with L-cysteine, α-ketoglutarate, and ferric iron prior to the assay. Two strains of *L. pneumophila* (Philadelphia 1 and SUMC 1), one strain of *L. dumoffii* (SUMC 1),

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and one strain of *L. feeleii* (CDC 1) were evaluated in this assay. Legionellae were grown for 24 to 48 h on buffered charcoal-yeast extract agar at 37°C in 5% CO_2.

Assay conditions. To harvest the amebae, culture tubes were vortexed vigorously, and then the suspended cells were centrifuged at 1,000 rpm for 10 min in the GP-7 rotor of a GPR centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The cells were resuspended in *A. castellanii* buffer [A.c. buffer; 4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3, pH 6.5] to a concentration of 2×10^5 cells per ml; 1 ml was then placed in each well of a 24-well tissue culture dish (Falcon 3047). The amebae were allowed to adhere to the wells and equilibrate for 1 h at 37°C before the bacteria were added, denoted as time point −3 h.

Approximately 5×10^9 CFU of legionellae per ml were suspended in sterile distilled water, and 10 μl of the bacterial suspension was added to each well containing amebae in 1 ml of A.c. buffer to give a ratio of approximately 200 legionellae per ameba. Legionellae were then added and incubation was carried out under different conditions over the course of 24 h. Bacterial invasion was permitted to continue for 1 hour at 37°C, after which time the buffer was aspirated and wells were washed once with warmed A.c. buffer and replaced with A.c. buffer containing 100 μg of gentamicin (Sigma). After a 2-h incubation at 37°C, which was determined to kill all extracellular legionellae (data not shown), the wells were again washed and fresh buffer without gentamicin was added; this time point was denoted as zero hours. At this point, the buffer was aspirated, and 1 ml of sterile distilled water was added followed by final lysis of amebae by drawing the suspension through a 27-gauge needle three times to break up the destabilized amebae. Detergents such as Triton X-100 could not be used to lyse the amebae because the *Legionella* cells were killed by exposure to even small amounts. Subsequent time points from 2 to 24 h after time zero were taken in triplicate by plating dilutions of the sample onto buffered charcoal-yeast extract agar, incubating at 37°C in 5% CO_2 for 4 days, and counting CFU to determine the number of intracellular bacteria.

Invasion assays with U-937 cells were performed in an identical manner except that the cells were raised in RPMI 1640 plus 10% fetal calf serum (GIBCO Laboratories, Life Technologies, Inc. Grand Island, N.Y.) and terminally differentiated to adherent macrophagelike cells by the addition of 10 ng of phorbol myristate acetate (Sigma) per ml for 2 days prior to the assay. The invasion assays with cytochalasin D and methylamine were performed as described above except 1 μg of cytochalasin D per ml and 100 mM methylamine (both from Sigma) were added 1 h prior to inoculation with Legionella bacteria. Each of these inhibitors was present for the duration of the assay in the same concentrations listed.

RESULTS

Growth of *Legionella* sp. in *A. castellanii*. The results of the quantitative *Acanthamoebae* assay performed under nutrient-limiting conditions demonstrated that all strains of *Legionella* spp. tested, with the exception of the avirulent *L. pneumophila* isolate obtained by more than 15 passages on supplemented Mueller-Hinton agar (3), were able to replicate in *A. castellanii* at 37°C. Therefore, we have confirmed in this host the differential ability of virulent versus avirulent *L. pneumophila* strains and *Legionella* species to enter eucaryotic cells and to replicate (4, 8, 9, 16) in a manner similar to that described in macrophages and monocytes (12, 17).

TABLE 1. Intracellular replication of legionellae in *Acanthamoeba* versus U-937 cells[a]

Legionella species (strain)	Net log growth		
	Growth at 37°C		Growth at room temp (*Acanthamoeba* cells)
	Acanthamoeba cells	U-937 cells	
L. pneumophila, virulent (Philadelphia 1)	+++	++	−
L. pneumophila, avirulent (Philadelphia 1)	−	−	−
L. dumoffii (SUMC 1d)[b]	+++	+++	−
L. feeleii (serogroup 1, CDC1f)	++	+	−

[a] The assay was performed as described in the text, and the extent of intracellular growth was determined after 20 h. +++, >2.0 net log increase; ++, 1.0 to 2.0 net log increase; +, <1.0 log increase; −, no growth. Calculations were made as the difference between time zero and 20 h, averaged from four experiments. Standard deviations for all data were $<\pm 0.2$ log.

[b] See reference 13.

In contrast to *A. castellanii*, U-937 cells were less effective at killing the wild-type *L. pneumophila* upon initial entry and were unable to kill any *Legionella* strain tested over the length of the assay (data not shown). The avirulent *L. pneumophila* strain was unable to grow in *A. castellanii* or U-937 cells. It should be noted that the data shown in Table 1 represent the average of four experiments, while Fig. 1 is a single representative experiment which was performed in triplicate; therefore, the absolute numbers of CFU varied between experiments.

Kinetics of growth in *A. castellanii*. The kinetics of intracellular replication of *Legionella* species in *A. castellanii* suspended in A.c. buffer demonstrated a lag period following entry and then exponential growth over a 10- to 20-h period, depending on the strain (Fig. 1). *L. pneumophila* had the shortest lag period (2 h), while the other two species had 4- and 6-h lag periods. A plateau phase was reached under these conditions, between 10 and 20 h, after which time we observed a decline in the number of viable *Acanthamoeba* cells. Under examination in an inverted microscope, unstained wells of the 24-well assay plate showed extracellular *L. pneumophila* and *L. dumoffii* appearing after 10 to 12 h, while *L. feeleii* did not break out of the cells until 24 h. Among the strains tested, the virulent *L. pneumophila* strain Philadelphia 1 replicated to the greatest extent over 20 h post-gentamicin treatment, while *L. dumoffii* SUMC 1 grew somewhat more slowly and *L. feeleii* CDC 1 grew most slowly at 37°C (Fig. 1). The kinetics of growth in U-937 cells were similar (Table 1), although net growth was less for all three *Legionella* strains in this cell line as compared with growth in *Acanthamoeba* cells. Using CFU measurements during exponential growth and the equations $N/N_0 = 10^{kt/2.303}$ and $g = \ln 2/k$ (where N_0 is the initial number of CFU, N is the final number of CFU, t is the time of exponential growth, and k is the calculated growth factor), the intracellular generation times (g) were calculated to be 56

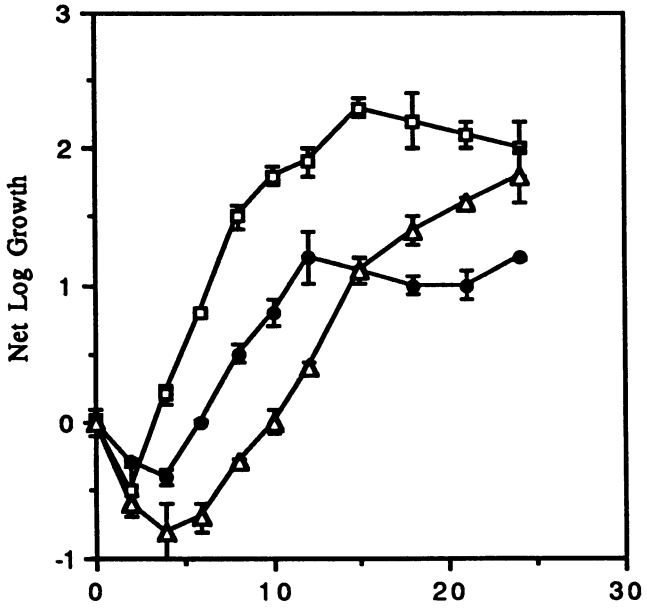

FIG. 1. Kinetics of growth of *Legionella* sp. in *A. castellanii*. These assays were performed in triplicate at 37°C as described in the text. Time zero was established to be the point following removal of gentamicin. These results are drawn from a single experiment and are representative of several assays. □, *L. pneumophila*; ●, *L. dumoffii*; △, *L. feelii*.

min for *L. pneumophila*, 88 min for *L. dumoffii*, and 115 min for *L. feeleii*.

Effect of nutrient limitation on intracellular growth. Several features of the assay differ from the system defined by Fields and coworkers (9, 14). First, in the model described here, neither extracellular *Legionella* nor *Acanthamoeba* cells were able to replicate because of nutrient limitation. To determine whether the presence of nutrients and the replication of the amebae during the assay affected the growth of the bacteria, the intracellular growth of virulent and avirulent *L. pneumophila* and *L. dumoffii* in *Acanthamoeba* cells was compared while the amebae were attached to tissue culture wells in PYG medium or A.c. buffer. Figure 2 shows that avirulent *L. pneumophila* were unable to grow in *Acanthamoeba* cells in either PYG or A.c. buffer, as was observed previously. The effect of assay medium on the intracellular growth of virulent *L. pneumophila* is shown in Fig. 3. Under conditions in which the starting inocula were similar, we observed that the amebae in A.c. buffer took up a larger portion of the inoculum than the amebae in PYG, but once inside, the bacteria grew to a similar extent. We noted that the number of bacteria measured at time zero was lower when the amebae were placed in PYG than when they were placed in A.c. buffer; however, the kinetics of growth were similar. Over the 21-h course of the assay (Fig. 3), the amebae suspended in PYG increased by 64% (1.1×10^5 to 1.8×10^5 per well), whereas the amebae neither grew nor decreased in A.c. buffer. All amebae were tested and confirmed to be viable (>98%) by eosin Y dye exclusion at the beginning and end of the assay. The effect of assay medium on the intracellular growth of *L. dumoffii* was similar to that seen in *L. pneumophila* (Fig. 4). In this assay using *L. dumoffii*, the amebae in PYG increased 73% over 24 h from 1.1×10^5 to 1.9×10^5 per well, while in A.c. buffer the amebae decreased 32% to 7.4×10^4 per well.

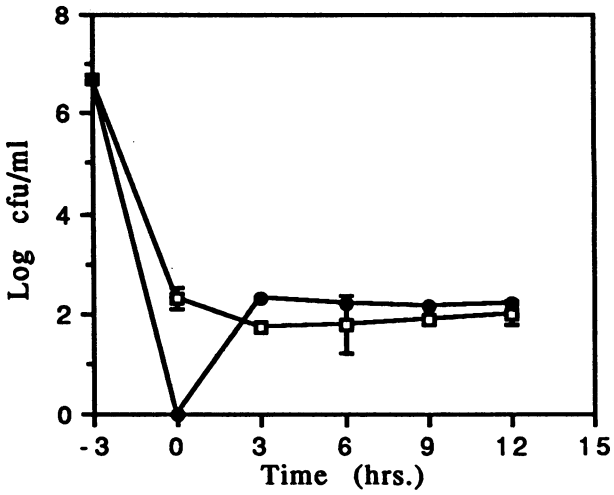

FIG. 2. Intracellular growth of avirulent *L. pneumophila* in PYG medium (□) or A.c. buffer (●). The assay was performed in triplicate at 37°C as described in the text except that PYG was used for half the samples in place of A.c. buffer in all cases. $t = -3$ was determined to be the time when bacteria were added. $t = 0$ is the point after the removal of gentamicin. The apparent drop to zero and then an increase to approximately 10^2 CFU between 0 and 3 h in A.c. buffer is most likely a sampling error due to the difficulty of detecting very small numbers of bacteria in each well.

Effect of MOI. To determine whether the level of bacterial infection of the amebae had an effect on the kinetics of the assay, several MOIs were tested with virulent *L. pneumophila*. Figure 5 shows that MOIs of approximately 10, 100, and 1,000 did not have a major effect on the rate of intracellular growth. The total numbers of bacterial CFU measured at the end of the assay were influenced by the MOI, however; a lower concentration of bacterial inoculum was reflected in a lower concentration of intracellular bacteria at the end of the assay period.

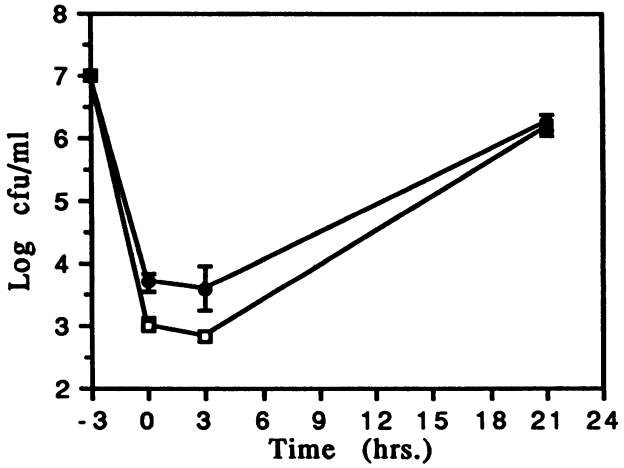

FIG. 3. Effect of nutrient limitation on intracellular replication of *L. pneumophila*. The assay was performed in triplicate at 37°C as described in the text except that PYG (□) was used for half the samples in place of A.c. buffer (●) in all cases. t = 3 was determined to be the time when bacteria were added. $t = 0$ is the point after the removal of gentamicin.

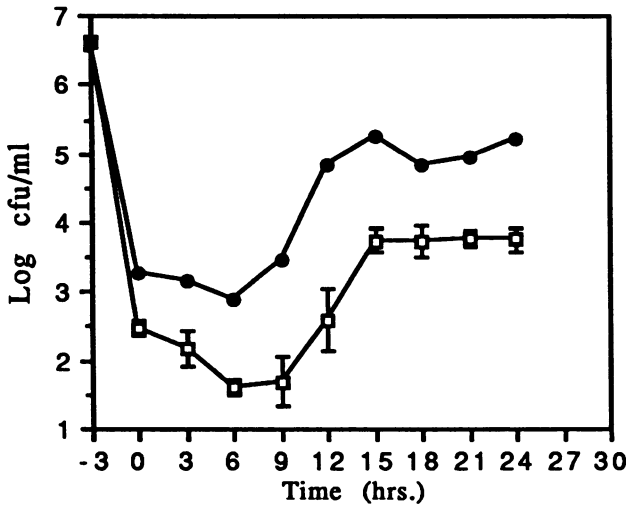

FIG. 4. Intracellular growth of *L. dumoffii* in PYG medium (□) or A.c. buffer (●). The assay was performed in triplicate at 37°C for those samples containing PYG as described in the text, replacing PYG for A.c. buffer in all cases. The assay was performed singly at 37°C as described for those samples containing A.c. buffer. $t = 3$ was determined to be the time when bacteria were added. $t = 0$ is the point after the removal of gentamicin.

Effect of temperature. The effect of temperature on intracellular survival and replication in *A. castellanii* was assessed by performing the invasion assay at room temperature (approximately 24°C) and at 37°C. Intracellular replication of all wild-type strains tested occurred when the assay was performed at 37°C. At room temperature, intracellular replication of neither virulent nor avirulent *L. pneumophila* occurred (data summarized in Table 1). The initial phase of bacterial uptake proceeded normally at room temperature, but viable bacteria could not be detected by 20 h postinfection. The amebae were not maintained beyond 24 h because of the nutrient limitation utilized to prevent *Acanthamoeba* growth; therefore, it is possible that small numbers of legionellae could have survived and replicated beyond this point. Subsequent intracellular replication was not influenced by the temperature under which the bacteria were grown prior to infection. Thus, we observed that the avirulent *L. pneumophila* strain was unable to replicate in amebae at room temperature, regardless of the temperature at which the assay was performed. Growth of wild-type *L. pneumophila* at room temperature similarly had no effect on the observable lag period or change in ability to replicate intracellularly.

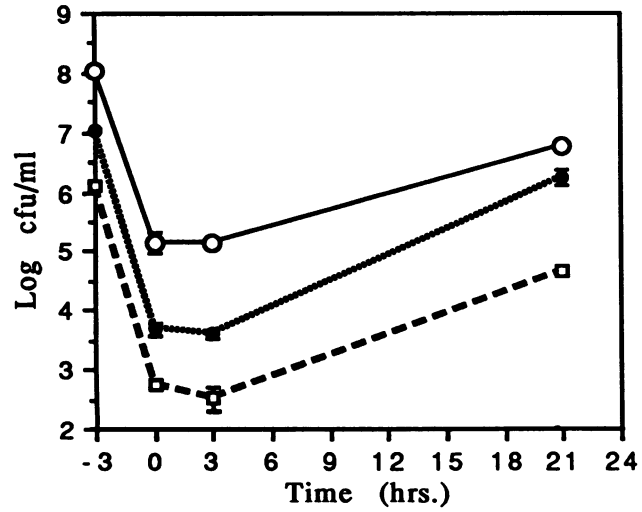

FIG. 5. Effect of MOI on intracellular growth of *L. pneumophila* in A.c. buffer. The assay was performed in triplicate at 37°C as described. MOIs were determined to be 10 (□), 100 (●), and 1,000 (○) by counting colonies on buffered charcoal-yeast extract agar. $t = 3$ was determined to be the time when bacteria were added. $t = 0$ is the point after the removal of gentamicin.

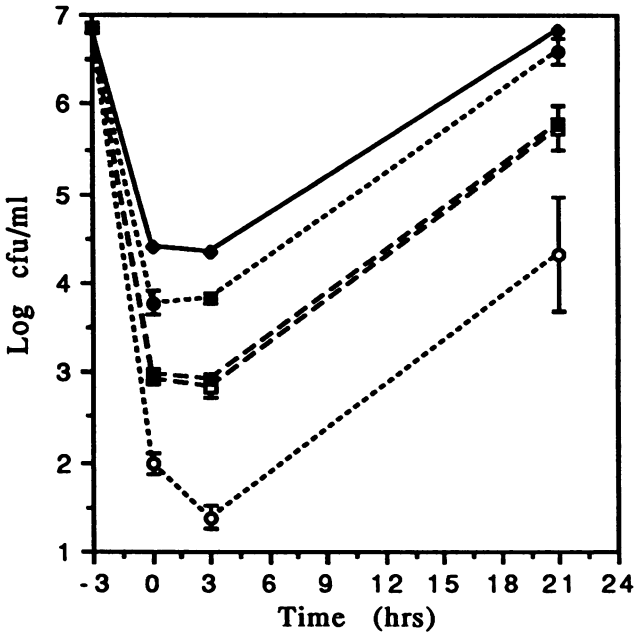

FIG. 6. Effects of methylamine and cytochalasin D added before or after inoculation with *L. pneumophila* on growth in *A. castellanii*. The assay was performed in triplicate at 37°C as described in the text, and the MOI was approximately 100. Methylamine at 100 mM and 1 μM cytochalasin D were added to a portion of the wells either 1 h before the addition of bacteria or at the time of gentamicin treatment. The inhibitors were present for the duration of the assay. ◆, *A. castellanii*; ○, methylamine added before bacteria; ●, methylamine added after bacteria; □, cytochalasin D added before bacteria; ■, cytochalasin D added after bacteria.

Effect of cytoskeletal inhibitors. The effects of methylamine and cytochalasin D on intracellular bacterial growth were addressed to determine whether these respective inhibitors of adsorptive pinocytosis and endocytosis altered the ability of *Legionella* spp. to enter or to replicate in *Acanthamoeba* cells. Figure 6 shows that, while neither inhibitor prevented *L. pneumophila* from replicating, 1 μg of cytochalasin D per ml significantly lowered the numbers of bacteria measured at the initial time points. Microscopic observation of the amebae treated with the inhibitors showed that cytochalasin D caused the amebae to round up and to lift off the culture dish, making them prone to washing away. This effect was corroborated by the 10-fold decrease (2.0×10^4 per well) in the number of amebae compared with the untreated control (2.15×10^5 per well) when cytochalasin D was added. Methylamine at 100 mM did not have a significant impact on growth; however, entry was greatly reduced when methyl-

amine was added prior to inoculation with bacteria. Adding methylamine 1 h after the bacteria reduced the initial CFU slightly, but subsequent growth was not affected. The number of viable amebae treated with methylamine remained constant throughout the experiment.

DISCUSSION

The model of intracellular growth presented here differed from others described previously (4, 9, 16, 17) in that the initial period of entry, lag phase, and exponential growth were clearly discernible. These other models extended bacterial growth up to 1 week and did not reveal the initial events that are detectable in our assay. In addition, the other models, using protozoa, macrophages, or tissue culture cell lines, did not restrict the growth of the host cells. We observed that nutrient limitation increased the uptake of legionellae by *A. castellanii* and suggest that A.c. buffer more closely mimics the conditions in aquatic environments where nutrients are very dilute. The early events of *Legionella* entry and intracellular replication are especially interesting for the reason that growth of wild-type strains appeared to be dependent on entry alone in our assay and the kinetics of growth were similar once the bacteria penetrated the host cell.

The ability of legionellae to grow in a wide variety of phagocytic cells has been documented. Explanted macrophages from humans and other animals (5, 6, 13) as well as the human macrophagelike cell line U-937 (17) support the growth of *L. pneumophila* but are not able to kill wild-type bacteria once intracellular. Treating macrophages or U-937 cells with cytochalasin D effectively inhibits the entry of *L. pneumophila* (6, 14), which consequently prevents replication of this facultative intracellular organism. This evidence suggests that legionellae enter macrophages and monocytes by normal endocytosis. In the protozoa *Acanthamoeba* and *Hartmanella* species, however, our model and another described by King et al (14) demonstrate that cytochalasin D probably has little effect on entry or replication of *L. pneumophila*. Some reduction of uptake was observed in our model since cytochalasin D caused the amebae to lift off the tissue culture plate and to wash away during the assay, reducing the number of target cells, which subsequently led to a reduction in final concentration of intracellular Legionella spp. This effect was not seen in the *Hartmanella* model (14) because the assay was performed on amebae suspended in growth medium which was not changed during the experiment, and thus no loss of amebae occurred. The ability of *L. pneumophila* to grow in *Hartmanella* and U-937 cells was shown previously to be inhibited by 100 mM methylamine (14), although we did not observe this. In the early stages of entry and replication documented in our model, methylamine reduced entry of *L. pneumophila* when added 1 h prior to bacterial inoculation; the ability of *L. pneumophila* to replicate in our assay was not inhibited by methylamine whether the compound was added before or after inoculation with bacteria. The discrepancy of these observations may be a result of the time course of the two assays. Methylamine may reduce uptake sufficiently in the *Hartmanella* model that intracellular replication was prevented over the course of 7 days, while in our *Acanthamoeba* model, the small numbers of *Legionella* spp. that entered in the presence of methylamine underwent a normal round of replication during the 24-h assay period.

The experimental results described here point to some general features of *Legionella* growth inside amebae. The presence of PYG, the MOI, and the adsorptive pinocytosis inhibitor methylamine all influence the number of bacteria taken up by the amebae. The effect of PYG appears to be that of lowering the rate of bacterial uptake, presumably by decreasing phagocytosis. Alternatively, the amebae in the rich PYG medium may be better able to kill intracellular bacteria. Once *Legionella* spp. were internalized, however, they were able to undergo unrestricted replication under all conditions tested except under lowered temperature. Therefore, given the appropriate temperature, all wild-type *Legionella* strains tested were able to replicate in this assay. Unlike other studies that show an increased ability of *L. pneumophila* grown at 25°C to cause disease in guinea pigs and to associate with macrophages (5), we noted no differences in uptake and growth of bacteria grown at room temperature (approximately 24°C) versus 37°C. On the other hand, the temperature at which the assay was conducted determined the ability of *Legionella* spp. to replicate in *Acanthamoeba* cells; at room temperature the internalized bacteria were killed, and at 37°C bacteria grew exponentially. Therefore, the ameba-*Legionella* interaction may have several outcomes depending on the temperature, with amebae phagocytizing and killing bacteria more readily at lowered temperatures and legionellae successfully outgrowing the host at raised temperatures. Temperatures that favor rapid growth of *L. pneumophila* (32 to 42°C [23]) in amebae could result in elevated numbers of bacteria being dispersed through systems carrying warm or hot water. Exposure to large numbers of *Legionella* spp. in hot-water sources such as shower heads (2) and hot-water systems (20, 23) has been correlated with outbreaks of Legionnaires' disease and Pontiac fever, especially when aerosols are formed. On the basis of our model, we propose that in warm-water conditions amebae serve as an important reservoir of intracellular legionellae and also could provide a continuous supply of extracellular bacteria in the water.

The intracellular growth rate was dependent on the species of *Legionella* and was easily calculated from the data obtained with this assay. In these experiments, the reduced intracellular growth rates of the species tested correlated with their relative virulence for humans, although this may be coincidental (7). The inability of the avirulent *L. pneumophila* variant to replicate intracellularly in amebae under nutrient-rich and nutrient-limiting conditions corroborates the results obtained by others with a different model system (12). This is consistent with observed lack of virulence in animal models, yet neither a genetic nor a biochemical basis for this has been discovered. Because the stages of early intracellular events can be studied in this assay, the genetic basis of avirulence could be approached more easily without resorting to cumbersome animal models.

Overall, this assay lends itself to a detailed study of intracellular replication of legionellae. Since a one-step growth curve in which *Legionella* spp. underwent a nearly synchronous life cycle for the duration of the assay could be obtained, this feature should allow us to address the molecular events that occur during each phase of growth. It will be especially interesting to study the events that occur during the initial lag phase of bacterial growth and, in particular, to examine the synthesis of bacterial proteins involved in pathogenesis and the regulation of expression during this period. In addition, it should now be possible to confirm the finding that motility phase variation occurs during intracellular growth (19). Finally, the comparison of the behavior of wild-type *Legionella* strains and isogenic mutants in *Acanthamoeba* and U-937 cells using this model may provide

additional information about the bacterial genes necessary for invasion, survival, and replication in each host.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

An intracellular growth assay was performed with 100 mM NH_4Cl (Sigma) in place of methylamine and showed no significant inhibition of *L. pneumophila* growth. This is evidence that the effect of methylamine was not due to the prevention of endosome acidification, since this is the primary result of ammonium chloride treatment.

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