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# Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: Uptake by Coiling Phagocytosis and Inhibition of Phagosome-Lysosome Fusion

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***Legionella pneumophila* is a facultative intracellular parasite able to survive within both human monocytes and amoebae. We have demonstrated that processing of *L. pneumophila* by the free-living amoeba *Acanthamoeba castellanii* shows many similarities to the processing of *L. pneumophila* by monocytes. These similarities include uptake of *L. pneumophila* by coiling phagocytosis and the subsequent confinement of *L. pneumophila* in a ribosome-studded phagosome. In addition, as in monocytes, inhibition of lysosomal fusion with phagosomes containing *L. pneumophila* was detected in amoebae. With all clinical isolates, inhibition of phagosome-lysosome fusion correlated with virulence. However, with one of the environmental isolates tested, no significant difference in phagosome-lysosome fusion was observed between the virulent and avirulent forms. These results indicate that the avirulent form of this isolate differed from the virulent form in some other respect critical to intracellular survival. Therefore, intracellular multiplication of *L. pneumophila* within *A. castellanii* may not be solely dependent upon the inhibition of lysosomal fusion.**

*Legionella pneumophila* is a gram-negative bacterium commonly isolated from lakes, streams, potable water supplies, and cooling towers (7, 11, 27). In these aquatic settings, *L. pneumophila* cells are able to parasitize and replicate within free-living amoebae (1, 16, 28, 31, 35) and *Tetrahymena* spp. (2, 15, 33). Thus, these protozoa may serve as important reservoirs for the bacteria in an aquatic environment. *L. pneumophila* can be transmitted from the aquatic environment by aerosolization and may lead to Legionnaires' disease, a severe form of pneumonia.

In the human lung, *L. pneumophila* is an intracellular pathogen of alveolar macrophages (22, 36). Phagocytosis of *L. pneumophila* proceeds, although not exclusively, by an unusual uptake event termed coiling phagocytosis (19). After uptake, the bacterium is confined to a phagosome. From this site, the bacterium is able to recruit small vesicles, mitochondria, and ribosomes to the outer surface of the phagosomal membrane (22). Furthermore, *L. pneumophila* cells are able to inhibit both the acidification of the phagosomes (21) and the subsequent fusion of lysosomes with the phagosomes (18). It is believed that inhibition of both phagosomal acidification and lysosomal fusion may be critical for the intracellular survival and growth of *L. pneumophila* in monocytes. Recently, two loci, *icm* (6, 25) and *dot* (4, 5), were described in which mutations in either locus lead to defects in intracellular growth, recruitment of host cell organelles, and inhibition of phagosome-lysosome fusion.

We have noted numerous similarities in the host-parasite relationship between *L. pneumophila* and its proposed natural host, free-living amoebae, to the interaction between *L. pneumophila* and human monocytes (17–19). Uptake of *L. pneumophila* by coiling phagocytosis and inhibition of lysosomal fusion with phagosomes containing *L. pneumophila* are demonstrated to occur within *Acanthamoeba castellanii*. In comparing matched pairs of virulent and avirulent isolates of *L. pneumophila* in amoebae, we show that virulence for clinical strains correlated with significantly lower levels of phagosome-lysosome fusion. However, a matched virulent and avirulent pair of an environmental isolate showed no significant difference in the levels of fusion. Thus, inhibition of fusion may be only one of several factors important for the intracellular parasitism of *L. pneumophila* within *A. castellanii.*

**Organisms.** Axenic *A. castellanii* (ATCC 30010) cells were obtained from Paul Schultz, Department of Ophthalmology, University of Iowa, Iowa City, and cultured as adherent cells in peptone-yeast extract-glucose broth (PYG) (ATCC medium 354) at 35°C. Amoebae were grown to confluence by 48 h.

*L. pneumophila* serogroup 1 strain Philadelphia 2 (Phil 2 Lp) was obtained from the Centers for Disease Control, Atlanta, Ga. *L. pneumophila* serogroup 1 strain Philadelphia 1 (Phil 1 Lp) was obtained from Clifford Mintz, Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Fla. Clinical (474.038 and 474.039) and environmental (474.026 and 474.027) isolates of *L. pneumophila* were obtained from the University of Iowa Hospitals and Clinics, Special Microbiology Laboratory, Iowa City. All clinical and environmental strains of *L. pneumophila* were screened for growth on supplemented Mueller-Hinton agar (BBL Microbiology Systems) supplemented with 0.025% ferric citrate and 0.25% cysteine (SMH agar) to test for mixed populations of bacteria converted to the avirulent form (9). Virulent and avirulent cultures of *L. pneumophila* were derived as previously described (10) and were maintained on charcoal yeast extract agar (CYE) (13). UV irradiation of *L. pneumophila* was carried out by the procedure described by Carlton and Brown (8). Formalin treatment of *L. pneumophila* was carried out by the procedure described by Horwitz (17). A 100% killing of the UV-irradiated and formalin-treated bacteria was achieved, as determined by assaying for the CFU of the treated suspensions. *Proteus mirabilis* (ATCC 29906) was used as a control organism in the fusion assays and was grown on nutrient agar plates.

**Assay conditions.** The virulence of the *L. pneumophila* strains was determined by their ability to multiply intracellularly within amoebae. Intracellular growth of *L. pneumophila*

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within amoebae was measured by harvesting PYG-grown amoebae by brief incubation of the monolayer on ice followed by dislodgment of the cells by mechanical disruption. The cells were suspended at a concentration of $2.5 \times 10^5$/ml, and 1-ml portions were added to each well of a 48-well tissue culture dish (Costar 3548). The plates were incubated at 35°C for 30 min to allow adherence of amoebae. After incubation, the PYG was aspirated off and replaced with *A. castellanii* buffer (26) followed by incubation for 1 h.

*L. pneumophila* cells were suspended in sterile distilled water to a concentration of $10^9$ CFU/ml. A volume of 50 μl was added to the amoeba monolayers to obtain a bacterium/cell ratio of approximately 200 bacteria to 1 amoeba. The bacteria and amoebae were incubated together for a period of 1 h at 35°C. Extracellular *L. pneumophila* cells were removed by washing the infected amoeba monolayer three times with *A. castellanii* buffer. This procedure removed 99.999% of the extracellular *L. pneumophila* cells, as determined by colony counts. After the final wash, 1 ml of fresh buffer was added. To determine the number of intracellular *L. pneumophila* cells at various time points, *A. castellanii* buffer was replaced with sterile distilled water, and the amoebae were lysed by drawing of the suspension though a 27-gauge needle three times (26). The numbers of CFU for all time points were determined by plating of dilutions of the samples in triplicate onto CYE agar followed by incubation at 37°C for 4 days.

**Electron microscopy.** Samples of amoebae infected with *L. pneumophila* were processed at 0.5, 1, 4, 12, and 24 h and fixed in sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 1 h at 35°C. Samples were then washed in sodium cacodylate buffer three times and postfixed in 2% osmium tetraoxide for 1 h. After passage through a series of graded acetone washes, the samples were embedded in Spurr epoxy resin. Sections were cut, stained in uranyl acetate and lead citrate, and examined with a Hitachi H-7000 transmission electron microscope.

**Electron microscopy for phagosome-lysosome fusion.** Fusion between amoeba lysosomes and phagosomes containing bacteria was determined by assaying for the presence of the lysosomal enzyme acid phosphatase (30). The control organism used for these studies was *P. mirabilis*, because it is readily digestible by amoebae. Unless otherwise indicated, all assays were carried out 30 min after the addition of *L. pneumophila* or *P. mirabilis* to the amoebae. The infected amoebae were incubated for 1 h in ice-cold 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. Cells were then washed in ice-cold 0.1 M sodium cacodylate buffer four times, followed by 3 washes with 0.1 M acetate buffer (pH 5.0). Acid phosphatase was localized by incubating the samples in cytochemical media (0.1 M acetate buffer, 2 mM β-glycerolphosphate as the substrate, and 1.2% lead nitrate as the capture metal) for 1 h at 37°C. Controls included samples in control media lacking substrate. Samples were then washed with acetate buffer and cacodylate buffer, postfixed in 2% osmium tetraoxide for 1 h, dehydrated though a series of graded acetone washes, and embedded in Spurr epoxy resin. Sections were cut, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7000 transmission electron microscope. Phagosome-lysosome fusion at later time points was measured by observation of fusion of the secondary lysosomes with phagosomes as previously described (18), with the exception that cationized ferritin (F 7879) obtained from Sigma Chemical Co. (St. Louis, Mo.) was used instead of thorium dioxide.

**Statistics.** In comparing the 1- and 24-h time points of the intracellular growth of the virulent *L. pneumophila* strains, statistical significance was determined by the two-tailed Student *t* test. In comparing the phagosome-lysosome fusion data, statistical significance ($P < 0.05$) was determined by the two-sample hypothesis method.

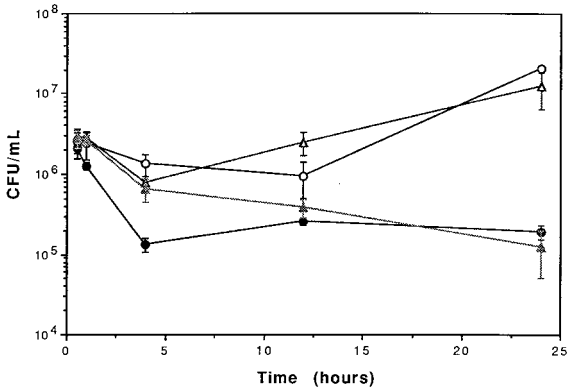


FIG. 1. Intracellular growth curves of the virulent and avirulent forms of Phil 2 Lp and the environmental isolate 474.026 in *A. castellanii* incubated in *A. castellanii* buffer at 35°C. Error bars represent standard errors of three independent experiments. ○, virulent Phil 2 Lp; ●, avirulent Phil 2 Lp; △, virulent 474.026; ▲, avirulent 474.026.

A difference in the ability of virulent and avirulent forms of Phil 2 Lp to grow within amoebae was observed (Fig. 1). Virulent Phil 2 Lp showed an initial lag period of 12 h, followed by growth over the next 12 h. In contrast, the avirulent Phil 2 Lp did not increase in numbers, but low levels of bacteria were recovered over the entire 24-h period of testing. Similar results were obtained with the virulent and avirulent forms of the Phil 1 strain of *L. pneumophila* (data not shown).

To demonstrate that the growth curves were not strain specific for the virulent and avirulent Phil 1 and 2 strains, comparisons between virulent and avirulent matched pairs of clinical (474.038 and 474.039) and environmental (474.026 and 474.027) isolates were made. In Fig. 1, intracellular growth of the virulent and avirulent forms of the environmental isolate 474.026 is shown as representative of that of the clinical and environmental isolates. Growth of the virulent form of isolate 474.026 was initiated by 4 h. In contrast, the avirulent derivative of 474.026 did not grow intracellularly but was recovered in low numbers over the entire 24-h period.

In studying the intracellular growth of *L. pneumophila*, we observed coiling phagocytosis to occur with the virulent form of Phil 2 Lp after 30 min of coincubation (Fig. 2). In addition, we have observed coiling phagocytosis occurring with formalin-treated Phil 2 Lp, the avirulent form of the Phil 2 Lp, and the clinical isolate 474.038 at similar time points (data not shown). Between 4 and 12 h after infection, the virulent form of Phil 2 Lp was found within ribosome-studded phagosomes (Fig. 3).

The results of the phagosome-lysosome assays are summarized in Table 1. *P. mirabilis* was readily digestible by *A. castellanii*, and 91% of the phagosomes containing *P. mirabilis* fused with amoeba lysosomes after 30 min of cocultivation. In contrast, only 7% of the phagosomes containing the virulent form of Phil 2 Lp showed the presence of acid phosphatase, indicating that the majority of phagosomes containing *L. pneumophila* were inhibited for fusion with lysosomes. Fusion was not delayed with avirulent Phil 2 Lp, since the levels of fusion at 6 h had not risen during testing for the presence of acid phosphatase (data not shown). To determine if inhibition of fusion was dependent on the presence of viable *L. pneumophila*, the intracellular fate of formalin-treated and UV-killed *L. pneumophila* cells ingested by *A. castellanii* was also deter-

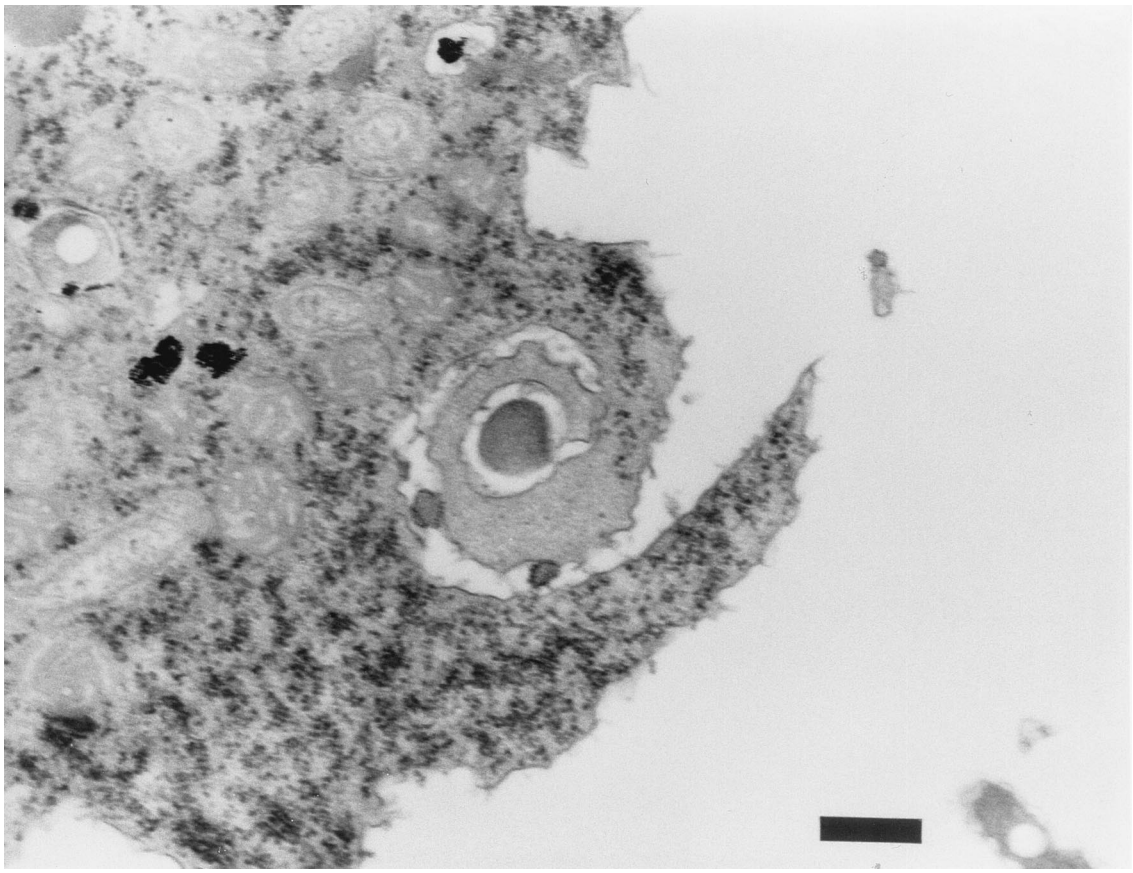

FIG. 2. Uptake of the virulent form of the Phil 2 strain of *L. pneumophila* by *A. castellanii* through coiling phagocytosis at 30 min of coincubation. Bar, 0.5 μm.

mined. With formalin-treated *L. pneumophila*, inhibition of phagosome-lysosome fusion was partially overcome, since the level of fusion rose from 7% to 35%. Similar results were observed with UV-irradiated *L. pneumophila*, in which fusion increased from 7% to 38%. The extent of fusion for virulent, avirulent, formalin-treated, and UV-irradiated *L. pneumophila* was significantly lower than the level of fusion obtained with *P. mirabilis* ($P < 0.001$).

Detection of acid phosphatase was used to measure lysosomal fusion at the early time points of *L. pneumophila* infection. To determine if phagosome-lysosome fusion was delayed, lysosomal fusion was also measured by preloading amoeba lysosomes with ferritin and determining if phagosomes containing bacteria fused with secondary lysosomes. The results of these experiments are listed in Table 2. Phagosomes containing *P. mirabilis* fused at a level of 93% with the secondary lysosomes. In contrast, only 9% of phagosomes containing virulent Phil 2 Lp were positive for the presence of ferritin, indicating that the majority of phagosomes containing *L. pneumophila* were inhibited for fusion with secondary lysosomes. Avirulent Phil 2 Lp showed partial inhibition of fusion, since 32% of the phagosomes containing these bacteria were positive for fusion. When virulent Phil 2 Lp was formalin treated, the levels of fusion increased from 9% to 36%. After infection for 6 h, the levels of fusion had not increased substantially for virulent Phil 2 Lp (13%), avirulent Phil 2 Lp (28%), or formalin-treated Phil 2 Lp (31%).

To determine if the differences in levels of fusion between virulent and avirulent *L. pneumophila* were strain specific, matched pairs of clinical and environmental isolates of virulent and avirulent strains of *L. pneumophila* were tested for phagosome-lysosome fusion by being measured for the presence of acid phosphatase after 30 min of coincubation (Table 3). Significant differences in fusion between the virulent and avirulent pairs of the environmental strain 474.027 (20% versus 53%) and clinical strains Phil 1 (21% versus 50%), 474.038 (22% versus 56%), and 474.039 (20% versus 54%) were observed. The differences in fusion between these virulent and avirulent pairs were similar to that observed with the virulent and avirulent forms of Phil 2 Lp. In contrast, the environmental isolate 474.026 showed no significant difference in the levels of fusion between the virulent and avirulent forms (40% versus 36% [$P < 0.5$]). The avirulent derivatives for all strains tested had levels of phagosome-lysosome fusion significantly lower than those of *P. mirabilis.*

In this study, we have investigated the host-parasite relationship between *L. pneumophila* and the free-living amoeba *A. castellanii*. A better understanding of this relationship is necessary, because protozoa serve as a reservoir for these bacteria in aquatic environments (1, 16, 28, 31, 35). In addition, protozoa may serve as an intracellular shelter protective against chlorine (23, 24) and biocides (3) added to plumbing systems to eradicate *L. pneumophila.*

Using electron microscopy, we have noted similarities in the intracellular growth of *L. pneumophila* in *A. castellanii* and in human monocytes. We have demonstrated the uptake of *L. pneumophila* by coiling phagocytosis in *A. castellanii*. We also observed internalization by coiling phagocytosis with virulent, avirulent, and formalin-treated Phil 2 Lp and with the clinical isolate 474.038. To our knowledge, coiling phagocytosis in pro-

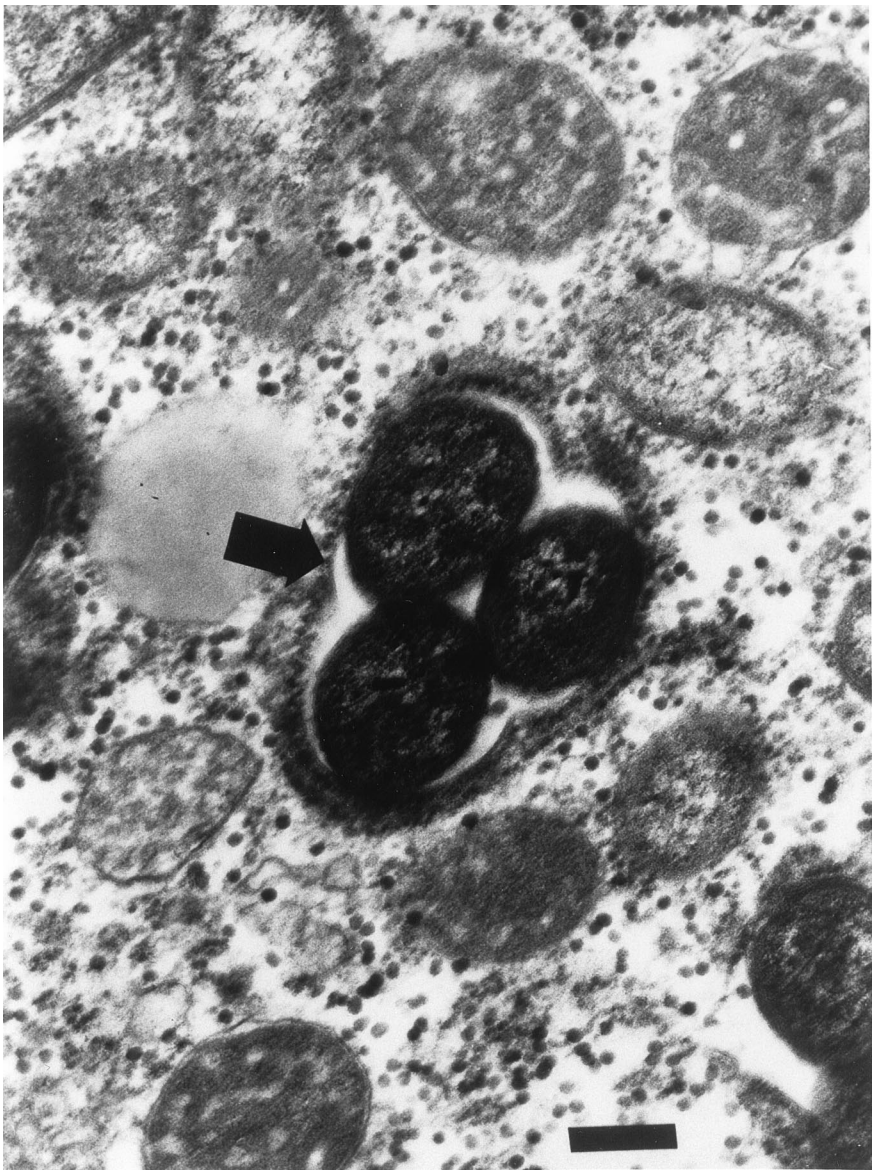

FIG. 3. *A. castellanii* infected with the virulent form of the Phil 2 strain of *L. pneumophila* for 12 h. Arrow indicates *L. pneumophila* located within a ribosome-studded phagosome. Bar, 0.25 μm.

tozoa has never been described. However, the significance of coiling phagocytosis in the pathogenesis of *L. pneumophila* in both macrophages and amoebae remains unclear. In macrophages, several studies have shown the uptake of *L. pneumophila* and other *Legionella* species to occur only by the conventional manner of phagocytosis (12, 29). More importantly, since coiling phagocytosis occurs with heat-killed, glutaraldehyde-killed, formalin-killed, and avirulent *L. pneumophila* (18, 20), differences in the intracellular fate of these bacteria must be attributed to some factor other than this unusual mechanism of uptake.

In our studies on the infection of *A. castellanii* with Phil 2 Lp, we observed *L. pneumophila* within ribosome-studded phagosomes at 4 h, as has been described to occur with monocytes (17) and at 12 h with *Hartmannella* (14) and *Naegleria* (28) spp. In macrophages, it has been demonstrated that the membranes surrounding the phagosomes containing *L. pneumophila* are derived from the rough endoplasmic reticulum, leading to the

TABLE 1. Acid phosphatase assay for phagosome-lysosome fusion of *L. pneumophila* Phil 2 strain and *P. mirabilis* within *A. castellanii*[a]

| Organism | % Fused[b] | *P* | |
|---|---|---|---|
| | | *P. mirabilis* vs *L. pneumophila* | Virulent *L. pneumophila* vs its derivatives |
| *P. mirabilis* | 91 (1) | | |
| *L. pneumophila* | | | |
| Virulent Phil 2 | 7 (2) | <0.001 | |
| Avirulent Phil 2 | 26 (8) | <0.001 | <0.05 |
| Virulent formalin treated | 35 (7) | <0.001 | <0.01 |
| Virulent UV killed | 38 (9) | <0.001 | <0.02 |

[a] Assay was carried out 30 min into infection.

[b] Data represent the means (± standard errors) of triplicate counts of 50 to 100 phagosomes on three different grids.

TABLE 2. Secondary lysosome labeling assay for phagosome-lysosome fusion for *L. pneumophila* Phil 2 strain and *P. mirabilis* within *A. castellanii*

| Organism | Time (min) | % Fused[a] | *P* | |
|---|---|---|---|---|
| | | | *P. mirabilis* vs *L. pneumophila* | Virulent *L. pneumophila* vs its derivatives |
| *P. mirabilis* | 30 | 93 (5) | | |
| *L. pneumophila* | | | | |
| Virulent Phil 2 | 30 | 9 (2) | <0.001 | |
| Avirulent Phil 2 | 30 | 32 (7) | <0.002 | <0.01 |
| Virulent formalin treated | 30 | 36 (2) | <0.001 | <0.001 |
| Virulent Phil 2 | 360 | 13 (6) | | |
| Avirulent Phil 2 | 360 | 28 (2) | | <0.05 |
| Virulent formalin-treated | 360 | 31 (2) | | <0.02 |

[a] Data represent the means (± standard errors) of triplicate counts of 50 to 100 phagosomes on three different grids.

presence of ribosome-studded phagosomes (34). To date, the significance of the accumulation of eucaryotic organelles in the intracellular survival of *L. pneumophila* remains to be determined.

One mechanism an intracellular parasite may use to evade the defenses of a host cell is to inhibit fusion of the phagosome containing the parasite with lysosomes. As shown in Table 1, phagosomes containing the virulent form of the Phil 2 Lp were inhibited for fusion with primary lysosomes of *A. castellanii*. When the virulent form was converted to an avirulent form by multiple passages on SMH agar, there was a significant increase in the levels of fusion. However, inhibition of fusion was only partially overcome, since the increase in fusion with avirulent Phil 2 Lp did not reach the levels seen with the control organism, *P. mirabilis*. Fusion with phagosomes containing avirulent Phil 2 Lp was not delayed in amoebae. After 6 h, levels of fusion had not risen when fusion was measured both by acid phosphatase and by secondary lysosomal labeling.

When the levels of fusion between virulent and avirulent matched pairs of clinical and environmental isolates were compared (Table 3), virulence correlated with inhibition of fusion for the clinical strains of *L. pneumophila* examined but not for the environmental isolate 474.027. Our results on the intracellular survival of *L. pneumophila* in *A. castellanii* are consistent with the results of others (29, 32) who have suggested that inhibition of fusion in macrophages infected with *L. pneumophila* may be only one of several factors responsible for the intracellular survival and multiplication of *L. pneumophila.*

TABLE 3. Phagosome-lysosome fusion for virulent and avirulent matched pairs of clinical and environmental isolates of *L. pneumophila*[a]

| Organism | Virulence | Avirulence | % Fused[b] | *P* | |
|---|---|---|---|---|---|
| | | | | *P. mirabilis* vs avirulent *L. pneumophila* | Matched virulent vs avirulent pairs of *L. pneumophila* isolates |
| *P. mirabilis* | | | 90 (3) | | |
| *L. pneumophila* | | | | | |
| Phil 1 | + | | 21 (7) | | |
| Phil 1 | | + | 50 (4) | <0.001 | <0.001 |
| 474.027 | + | | 20 (8) | | |
| 474.027 | | + | 53 (4) | <0.001 | <0.001 |
| 474.038 | + | | 22 (3) | | |
| 474.038 | | + | 56 (3) | <0.001 | <0.001 |
| 474.039 | + | | 20 (1) | | |
| 474.039 | | + | 54 (4) | <0.001 | <0.001 |
| 474.026 | + | | 40 (6) | | |
| 474.026 | | + | 36 (3) | <0.001 | <0.5 |

[a] Assay was carried out 30 min into infection, and result was determined by staining for acid phosphatase.

[b] Data represent the means (± standard errors) of triplicate counts of 50 to 100 phagosomes on three different grids.

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