

Passage through *Tetrahymena tropicalis* Triggers a Rapid Morphological Differentiation in *Legionella pneumophila*[∇]

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The intracellular bacterial pathogen *Legionella pneumophila* follows a developmental cycle in which replicative forms (RFs) differentiate into infectious stationary-phase forms (SPFs) in vitro and in vivo into highly infectious mature intracellular forms (MIFs). The potential relationships between SPFs and MIFs remain uncharacterized. Previously we determined that *L. pneumophila* survives, but does not replicate, while it transiently resides (for 1 to 2 h) in food vacuoles of the freshwater ciliate *Tetrahymena tropicalis* before being expelled as legionellae-laden pellets. We report here that SPFs have the ability to rapidly (<1 h) and directly (in the absence of bacterial replication) differentiate into MIFs while in transit through *T. tropicalis*, indicating that SPFs and MIFs constitute a differentiation continuum. Mutant RFs lacking the sigma factor gene *rpoS*, or the response regulator gene *letA*, were unable to produce normal SPFs in vitro and did not fully differentiate into MIFs in vivo, further supporting the existence of a common mechanism of differentiation shared by SPFs and MIFs. Mutants with a defective Dot/Icm system morphologically differentiated into MIFs while in transit through *T. tropicalis*. Therefore, *T. tropicalis* has allowed us to unequivocally conclude that SPFs can directly differentiate into MIFs and that the Dot/Icm system is not required for differentiation, two events that could not be experimentally addressed before. The *Tetrahymena* model can now be exploited to study the signals that trigger MIF development in vivo and is the only replication-independent model reported to date that allows the differentiation of Dot/Icm mutants into MIFs.

The natural history of *Legionella pneumophila* indicates that this gram-negative bacterium evolved as an intracellular pathogen of amoebae. In nature, as well as in human-made water systems, reproduction of *L. pneumophila* takes place primarily in amoebae (16, 31, 37). When *L. pneumophila* accidentally reaches the alveoli of susceptible humans, it may cause an atypical pneumonia known as Legionnaires' disease, characterized by the intracellular growth of *L. pneumophila* in alveolar macrophages (reviewed in reference 13). *L. pneumophila* thus behaves like (although it is not) an obligate intracellular pathogen, emulating its close relatives *Coxiella* spp. (40, 42) and the many *Legionella*-like amoebal pathogens (3, 10).

Key to the life cycle of obligate intracellular pathogens is their ability to alternate between a replicative form (RF), which produces intracellular progeny, and a nonreplicative form that is infectious and survives in the environment until a new host is found (36). *L. pneumophila* is no exception (20), and it alternates between an RF and a mature intracellular form (MIF) that is highly infectious to cells in culture and resistant to environmental stress (12, 21, 22; reviewed in reference 18). At the ultrastructural level, the RF of *L. pneumophila* shows a typical gram-negative envelope, a moderately

dense cytoplasm rich in ribosomes, and cell division. RFs are susceptible to environmental stress and exhibit poor infectivity (11, 30). In contrast, the MIF displays an altered and complex envelope architecture, an electron-dense cytoplasm with large inclusions, and no morphological evidence of cell division (15, 21).

In vitro, *L. pneumophila* grown in broth or on solid medium differentiates into infectious stationary-phase forms (11, 35) but does not form MIFs (21). Therefore, *L. pneumophila* RFs produce two differentiated forms, stationary-phase forms (SPFs) in vitro and MIFs in vivo. Although SPFs and MIFs share some traits, such as the ability to infect cells in culture, resistance to heat, and the presence of cytoplasmic inclusions, they show numerous differences: MIFs are 10- to 100-fold more infectious to HeLa cells than SPFs, have a unique envelope ultrastructure, have a very low basal respiration rate, and are resistant to antibiotics, high pH, and lysis by detergents (21). A plausible explanation for these observed similarities and differences between SPFs and MIFs is the possibility that SPFs represent a differentiation intermediate in the RF-to-MIF transition (21), a hypothesis that has remained experimentally unproven. Therefore, we set out to elucidate the developmental relationships between SPFs and MIFs, to better understand the process of *L. pneumophila* differentiation.

We have previously shown that *L. pneumophila* is efficiently ingested by the freshwater ciliate *Tetrahymena tropicalis* and transiently resides in the ciliate's food vacuoles for about 1 to 2 h before being expelled in the form of spherical pellets containing live legionellae (9). We also determined that during

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its transient residence in *T. tropicalis*, *L. pneumophila* does not replicate (9). Here, we report that *T. tropicalis* organisms feeding on SPFs expel live legionellae with all the morphological characteristics of MIFs, clearly indicating that SPFs have the ability to rapidly (<1 h) and directly (in the absence of bacterial replication) differentiate into MIFs, thus confirming that SPFs constitute differentiation intermediates in the RF-to-MIF transition. In addition, this study focuses on the morphology of the various *L. pneumophila* forms present in food vacuoles of *T. tropicalis* feeding on avirulent *dot* mutants, or regulatory mutants unable to produce normal SPFs in vitro, in an effort to identify some of the factors involved in MIF development.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *L. pneumophila* Philadelphia-1 virulent strain Lp1-SVir (streptomycin resistant) has been described previously (29). The *L. pneumophila* Philadelphia-1 virulent strain Lp02 (a transformable thymidine auxotroph) and its nonvirulent, salt-tolerant derivatives JV303 (*dotB* mutant) and JV309 (*dotA* mutant) were obtained from Ralph Isberg, Tufts University Medical School (8, 43). Lp02 derivatives MB445 (*rpoS::kan* carrying the empty vector pKB5*mobAΔ*) and MB434 (*letA-22-3* carrying the empty plasmid vector pMMB*GentmobAΔ*) were a gift from Michele Swanson, University of Michigan Medical School, as were the genetically complemented strains MB444 (*rpoS::kan* carrying the plasmid pRpoS⁺, which is pKB5*mobAΔ* harboring a wild-type copy of *rpoS*) and MB435 (*letA-22-3* carrying the plasmid pLetA, which is pMMB*GentmobAΔ* harboring a wild-type copy of *letA*) (5, 26, 27). All strains were kept as frozen stocks at -80°C in buffered yeast extract (BYE) broth (see below) with 7% dimethyl sulfoxide. Frozen stocks of virulent strains Lp02, Lp1-SVir, MB444, and MB435 were made from crude lysates of infected HeLa cells after intracellular growth had taken place (23). Frozen stocks of Lp1-SVir were routinely grown for 3 to 5 days at 37°C in a humid incubator, on buffered charcoal-yeast extract agar (BCYE) (39) supplemented with 100 µg/ml of streptomycin. Frozen stocks of Lp02 and its derivatives JV303 and JV309 were grown on BCYE agar containing streptomycin and thymidine (both at 100 µg/ml). Strains MB444 and MB445 were grown on BCYE containing streptomycin (100 µg/ml) and kanamycin (25 µg/ml), but without thymidine. Strains MB434 and MB435 were grown on BCYE containing streptomycin and thymidine (both at 100 µg/ml), kanamycin (25 µg/ml), and gentamicin (50 µg/ml). Bacteria from the 5-day growth on solid media were either harvested and used without subculture to feed *T. tropicalis* (see below) or subcultured to stationary phase (11) in BYE with the appropriate supplements, when needed. BYE had the same formulation as BCYE, but charcoal and agar were omitted. Before use in feeding experiments, the bacterial cells were washed in modified Tris-buffered Osterhout's solution (TBOS) (9) and resuspended in an appropriate volume of the same solution to achieve an optical density (read at 620 nm) of 1 unit, equivalent to ~10⁹ legionellae/ml.

Tetrahymena speciation. We previously identified our pellet-producing ciliate as a species of the genus *Tetrahymena* within the *T. mobilis*-*T. tropicalis* group, based on the DNA sequence of its gene encoding the small subunit rRNA (9). To determine the species, we used the DNA sequence of a fragment of the gene encoding the mitochondrial cytochrome *c* oxidase (CO1), as reported by Lynn and Strüder-Kypke (33). Total DNA was extracted by a modification of the method of Arroyo et al. (4). Briefly, ~10⁶ *Tetrahymena* cells were harvested by centrifugation (700 × *g*, 15 min) and washed once in 10 mM Tris, 1 mM EDTA, pH 8. The cell pellet was resuspended in 300 µl of lysis buffer (0.4% sodium dodecyl sulfate, 100 mM NaCl, 10 mM EDTA, 10 mM Tris, 0.02% dimethyl sulfoxide, 2.0% sucrose, pH 7) and incubated 1 h at 60°C, followed by 5 min at 93°C. Total DNA was then extracted with phenol-chloroform followed by an additional extraction with chloroform. After precipitation in ethanol at -20°C, the DNA pellet was solubilized in 16 MΩ · cm MilliQ water (Millipore) and used as template in subsequent PCRs. PCR amplification of the CO1 fragment was performed with forward primer 288 and reverse primer Fo1B as previously described (33), but using a slightly modified PCR amplification protocol, as follows: initial denaturation at 95°C for 5 min; 5 amplification cycles at 95°C for 45 s, 45°C for 1.5 min, and 72°C for 2 min; 30 cycles at 94°C for 45 s, 51°C for 2 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. The amplification product was visualized by ethidium bromide staining after agarose gel electrophoresis and purified using the Marligen/Biosciences Inc. rapid gel ex-

traction kit. DNA sequencing was performed at the Instituto de Investigaciones Biomédicas (Mexico City) using a Genetic Analyzer 310 (PE Applied Biosystems). The sequencing results indicated that our isolate belongs to the *T. tropicalis* species (33).

Ciliate cultures. The *T. tropicalis* isolate used here was originally isolated from a cooling tower biofilm and maintained in axenic culture according to the procedures outlined by Elliot (14), as detailed elsewhere (9). Before use in feeding studies, *T. tropicalis* cells were gradually transferred from their plate count broth (PCB) (Difco) growth medium (by sequential pelleting at 700 × *g* for 10 min and resuspension) into increasing concentrations of modified TBOS. Modified TBOS contained (in mg/liter) NaCl (420), KCl (9.2), CaCl₂ (4), MgSO₄ · 7H₂O (16), MgCl₂ · 6H₂O (34), and Tris base (121) and was sterilized by filtration in a bottle-top 0.45-µm filter (Nalgene) after adjusting the pH to 7. The intermediate concentration steps used for the PCB-to-TBOS transition were PCB 50%-TBOS 50%, PCB 25%-TBOS 75%, and PCB 12.5%-TBOS 87.5%.

Feeding experiments. All feeding experiments were performed in six-well plates (Falcon Plastics) at room temperature (22 to 25°C) with 1.5 × 10⁶ *Tetrahymena* cells and ~5 × 10⁸ *L. pneumophila* cells per well resuspended in 3 ml of modified TBOS. Tris-buffered Osterhout's solution. Samples were taken at different times for electron microscopy by pelleting the ciliate-bacteria mixture at 700 × *g* for 10 min in 15-ml conical tubes (Falcon Plastics) and removing the supernatant. To produce samples enriched in expelled pellets, the ciliate-bacteria mixture was incubated overnight (16 to 18 h) at room temperature and then centrifuged at 700 × *g* for 10 min in 15-ml conical tubes. The live ciliates were then allowed to swim back into suspension before removing the supernatant. This operation was repeated three times, after which the recovered sample almost exclusively contained aggregated, ciliate-expelled pellets containing legionellae.

HeLa cells. HeLa cells were cultured at 37°C, 5% CO₂ in 25-cm² flasks (Falcon Plastics) containing 7 ml of complete minimal essential medium (MEM) as previously described (23). For infection, HeLa cells from flasks were trypsinized, transferred to six-well plates at a ratio of 10⁶ cells per well, and cultured until confluent monolayers were formed. An inoculum of 10⁸ bacteria/well was then added and incubated overnight (~16 h) to allow bacterial invasion and initiation of intracellular growth. The infected cells were then washed three times with warm PBS and treated with gentamicin (100 µg/ml) for 2 h in MEM. The MEM with gentamicin was removed with two washes of warm PBS and replaced with fresh MEM without antibiotics. Then, HeLa cells were further incubated (for up to 2 to 3 days) and observed in a 1X71 Olympus inverted microscope until legionellae-laden vacuoles were observed. Then, the infected cultures were air dried for Giménez staining and/or harvested to be prepared for electron microscopy.

Light microscopy. Ciliate cultures and HeLa cells were routinely monitored in a 1X71 Olympus inverted microscope equipped with phase contrast. Giménez staining with carbol fuchsin as primary stain and malachite green as secondary stain was performed as described by McDade (34) except that the malachite green solution was used at 4% instead of 0.8%. The application of Giménez staining to the detection of MIFs has been described elsewhere (21). Giménez-stained preparations were observed in an Olympus BX61 upright microscope equipped with an Evolution QEI monochrome digital camera (Media Cybernetics, San Diego, CA). Micrographs were captured as TIFF files using Image Pro software (Media Cybernetics). Direct counts of HeLa cells in suspension were done in a Neubauer hemocytometer, as was the enumeration of ciliates fixed with Lugol's iodine (9).

Transmission electron microscopy. Samples of *Tetrahymena* cells feeding on *L. pneumophila*, taken at different times, were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated with ethanol, and embedded in epoxy resin for thin sectioning, followed by standard staining in uranium and lead salts, as described previously (15). Thin sections were observed in a JEOL JEM-1230 transmission electron microscope equipped with a Hamamatsu ORCA-HR high-resolution (2,000 by 2,000 pixels) digital camera, and images were saved as TIFF files. To enumerate the various morphological forms of *L. pneumophila* present in our samples, we were careful to include ultrathin sections from at least two separate experiments and record images from at least 50 sectioned cells from each experiment. While quantification was not the foremost aim of this study, micrographs were purposely selected to represent the full scope of our observations, and counting of particular parameters was carried out on the actual micrographs, rather than at the electron microscope.

Immunoblotting to detect MagA. Approximately 10⁶ ciliates resuspended in 30 ml of modified TBOS were fed with ~3 × 10¹⁰ *L. pneumophila* Lp1-SVir cells in 25-cm² cell culture flasks. After a feeding period of 3 h, the ciliates were separated from free bacteria by filtration through SCWP 025 00 Millipore (Bedford, MA) hydrophilic membranes with 8.0-µm pores. The ciliates remaining on the

filter were washed once by passing ~30 ml of modified TBOS through the filter and then resuspended in 30 ml of modified TBOS by gentle pipetting, followed by a static 5-min incubation to allow ciliates to freely swim into suspension. Samples (3 ml total) were taken at various times and split to perform legionellae CFU counts and immunoblotting. Samples for CFU counts (1 ml) were placed in a 1.5-ml microcentrifuge tube and centrifuged at $10,000 \times g$ for 1 min. To lyse ciliates, as well as disperse and break up legionella-laden pellets, the centrifugation pellet was quickly resuspended in 50 μ l of 0.5% Triton X-100 in sterile deionized water (dH₂O) with vigorous pipetting. Then, 450 μ l of dH₂O was added, followed by vigorous vortexing for 1 min. The sample was brought to 1 ml by adding 50 μ l of 0.5% Triton X-100 in dH₂O and 450 μ l of dH₂O, before performing serial dilutions. Aliquots (100 μ l) of the 10^2 to 10^6 dilutions were spotted on BCYE plates and incubated at 37°C for 4 to 5 days before counting colonies. Samples for immunoblotting (2 ml) were centrifuged at $10,000 \times g$ in two 1.5-ml microcentrifuge tubes for 1 min, and the centrifugation pellet was kept frozen until results from the bacterial CFU counts were obtained. Loading onto 12% acrylamide gels was adjusted based upon CFU/ml values for each sample, so that the equivalent to 5×10^4 CFU was loaded per lane in 15 μ l of sample buffer. Solubilized proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a minigel vertical slab apparatus (Bio-Rad), and the separated proteins were transferred onto a nitrocellulose membrane in a Transblot apparatus (Bio-Rad). The nitrocellulose membrane was then immunostained with a hyperimmune rabbit serum raised against purified MagA protein, as described previously (22, 28). The immunostained membrane was scanned to produce a TIFF file that was then analyzed with Gel-Pro Analyzer version 4.5 software (Media Cybernetics, Silver Spring, MD), to obtain integrated optical density (IOD) values (in arbitrary units) for the immunostained MagA bands, using the single-band analysis tool. In a second experiment, we compared the amount of MagA present in Lp02 MIFs purified from *Tetrahymena* pellets, Lp02 MIFs purified from HeLa cells, and Lp02 SPFs collected by centrifugation from a BYE culture and washed once in dH₂O. A suspension enriched in legionella-laden pellets (produced as described above under "Feeding experiments") was mechanically disrupted by forcefully passing it 20 times through a 27-gauge needle. Released free MIFs and undisturbed small pellets were further fractionated in a continuous Percoll density gradient, produced as described previously (23). Free MIFs were collected from the bottom (most dense) layer. MIFs from HeLa cells were also purified in a continuous Percoll density gradient as previously reported (23). At least $\sim 2 \times 10^9$ bacteria (estimated by optical density) were dissolved in 60 to 100 μ l of 2 \times Laemmli sample buffer, and the amount of total protein was then determined by the detergent-compatible D_C protein assay (Bio-Rad). Loading onto 12% acrylamide gels was targeted to 20 μ g of total protein per lane, and samples were processed for immunoblotting as described above. Before immunostaining with the MagA-specific antibody, the nitrocellulose membrane was stained with Ponceau-S and a digital image of the stained membrane was obtained as a TIFF file in an Epson ES 1200C scanner. After immunostaining, the IOD of MagA bands was obtained using a Gel-Pro Analyzer. These IOD values were then corrected for loading and efficiency of transfer in relation to the IOD of four arbitrarily selected and apparently constant bands from the Ponceau-S-stained nitrocellulose membrane.

RESULTS

Ultrastructural features of food vacuoles and vacuole maturation. Our pellet-producing *Tetrahymena* sp. was speciated as *T. tropicalis*, based on the mitochondrial cytochrome *c* oxidase gene sequence (33). As previously reported (9), food vacuoles of diverse morphology and stage of maturation were present concurrently in each ciliate, due to constant food vacuole generation in actively feeding *T. tropicalis* cells (Fig. 1). Based on fine structural observations, it was clear that normal trafficking (or maturation) of the *T. tropicalis* food vacuoles was accompanied by a striking remodelling of the vacuoles. It has not escaped our notice that the morphological resemblance of this remodelling process and the early maturation of *L. pneumophila*-containing vacuoles in mammalian host cells suggests a common underlying mechanism. In the initial stages of feeding, nascent food vacuoles were easily identified at the profusely ciliated cytopharynx. These early vacuoles were characterized by a loose membrane defining a spacious interior

(Fig. 1A and B). Based on previously reported pulse-chase experiments with fluorescent legionellae (9), internal vacuoles detached from the cytopharynx began to appear as early as 5 min after addition of the bacterial inoculum, indicating that early vacuoles are short-lived. Mid-stage maturation was depicted by vacuoles bound by a membrane that closely followed the contour of the contained legionellae and the first appearance of membranous material in the vacuole (Fig. 1C and D). Late-stage vacuoles were identified by their roughly spherical shape, a smooth membrane profile, tightly-packed bacteria and membrane remnants, and a characteristic "halo," or space, between the condensed content and the food vacuole's membrane (Fig. 1E and F). Together, the mid- and late vacuole maturation stages must be completed in 50 to 60 min, because the first pellets expelled by actively feeding *T. tropicalis* are seen ~1 h after addition of the bacterial inoculum (9). After 1 h of active feeding, each ultrathin-sectioned ciliate presented more late-stage vacuoles than mid-stage ones, suggesting that mid-stage maturation may be shorter than late-stage vacuole maturation. While our vacuole progression scheme is not strictly based on the time course, it closely agrees with early reports on *Tetrahymena pyriformis* which described the "dehydration" of the newly formed vacuole and the condensation of the contained bacteria into a tightly packed sphere (see references 14 and 38 and references within).

Morphological features of vacuole-contained legionellae. Micrographs of early-, mid-, and late-stage legionellae-containing food vacuoles did not show ultrastructural evidence of bacterial cell division, suggesting that the legionellae in each food vacuole represent a captive, isolated, and nonreplicating population. These bacteria, even within early- and mid-stage vacuoles, showed several ultrastructural features typical of MIFs (Fig. 2). The MIF-associated features (previously detailed in references 15 and 21) included prominent cytoplasmic inclusions, thick-layered envelopes, a dark cytoplasm, irregular shapes, and an unapparent periplasm (Fig. 2). In each food vacuole bacteria seemed to be experiencing the same morphological changes at the same time (Fig. 1 and 2), suggesting synchronicity. Furthermore, the legionellae in vacuoles and free expelled pellets projected a bright red color after Giménez staining (not shown), another characteristic of MIFs (21). In addition, lysates obtained from ciliates fed with strain Lp1-SVir confirmed enhanced levels of MagA (Fig. 3), a protein previously reported to be a marker of MIF formation for Philadelphia-1 *L. pneumophila* strains (28). To avoid any potential skewing of our MagA immunoblotting results by differences in culturability (since the gel shown in Fig. 3 was loaded to equivalent CFU per lane), we ran a second immunoblot assay to compare purified Lp02 MIFs to purified Lp02 SPFs in which the gel's lanes were loaded based on total protein. The IOD values for the immunostained MagA bands in this second experiment were 212 for SPFs and 938 for the MIFs purified from *T. tropicalis*-expelled pellets. The IOD value of the immunostained MagA band of Lp02 MIFs purified from HeLa cells (used as a reference for enhanced expression of MagA [21]) was 547. From these results we concluded that *L. pneumophila* rapidly differentiates into MIFs in response to the food vacuole environment of *T. tropicalis*, in a rather synchronous process that is unlinked to bacterial replication.

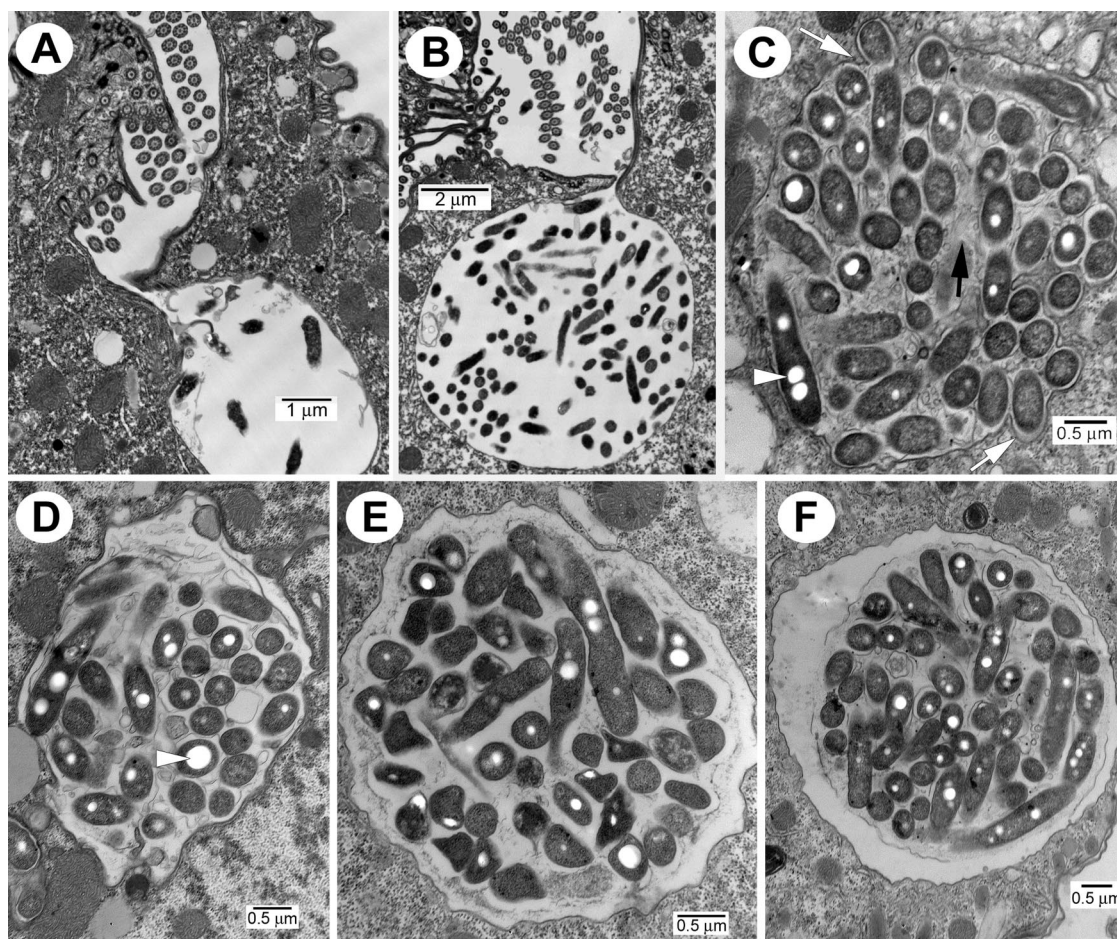


FIG. 1. Estimated reconstruction of the morphological progression of *Legionella*-containing food vacuoles in *T. tropicalis*, as depicted in the electron microscope. (A and B) Development at the ciliate's cytopharynx of early spacious vacuoles with a smooth membrane and spherical shape. (C) Mid-stage vacuole bound by a circuitous membrane that closely follows the outline of the contained peripheral bacteria (white arrows) and showing the first evidence of bacterial cell degradation resulting in accumulation of membrane material (black arrow). Large bacterial inclusions, characteristic of MIF development, are indicated by the white arrowhead. (D) Mid-stage vacuole in which the lining membrane appears detached from the contained peripheral bacteria and has begun to acquire a smoother contour. The arrowhead points to a prominent inclusion. (E) Late-stage vacuole with a smooth lining membrane and a defined spherical shape. (F) Late-stage spherical-shaped vacuole depicting a "halo" or space between the smooth vacuolar membrane and the tight concentration of bacteria (wrapped by membrane fragments) similar in appearance to expelled pellets.

***rpoS* and *letA* mutants are extensively digested in *T. tropicalis*.** The sigma factor RpoS and the response regulator LetA regulate the differentiation of *L. pneumophila* RFs into SPFs, mainly in response to amino acid starvation *in vitro* (reviewed in references 19 and 35). Therefore, *rpoS* and *letA* mutants do not produce normal SPFs (26, 27). To check whether RpoS and LetA are also involved in MIF formation within *T. tropicalis*, the ciliates were fed the Lp02 derivatives MB445 (*rpoS* mutant) and MB434 (*letA* mutant) and their in vacuole ultrastructure was examined.

Unexpectedly, both regulatory mutants were digested to a large extent by *T. tropicalis*, and abundant membranous material was typically present in both food vacuoles and expelled pellets (Fig. 4). The accumulated membrane has been previously shown to be bacterial in origin and to represent outer membrane remains of digested legionellae (9). However, survivor *rpoS* mutants seemingly depicted morphological characteristics typical of MIFs, as shown in Fig. 4A, but their very low

abundance did not allow us to make reliable observations. The number of *letA* mutant cells that resisted digestion was negligible. In fact, nearly all expelled pellets of the *letA* mutant contained no bacterial cells (Fig. 4E). Thus, the characterization of the *rpoS* and *letA* mutants' ultrastructures in *T. tropicalis* was compromised by the very low numbers of morphologically intact cells found.

To confirm that the inability to survive (and consequently differentiate) in *T. tropicalis* was indeed attributable to the mutated *rpoS* and *letA* genes, the mutants were genetically complemented *in trans* with plasmid-borne wild-type copies of *rpoS* and *letA*, respectively. The complemented mutant strains MB444 (wild-type *rpoS*) and MB435 (wild-type *letA*) recovered both the ability to resist in vacuole digestion and differentiate into MIFs and consequently were able to produce MIF-laden pellets in *T. tropicalis* (Fig. 4C and F). However, it is important to note that the recovery of wild-type MIF morphological traits in the genetically complemented strains was not complete, so

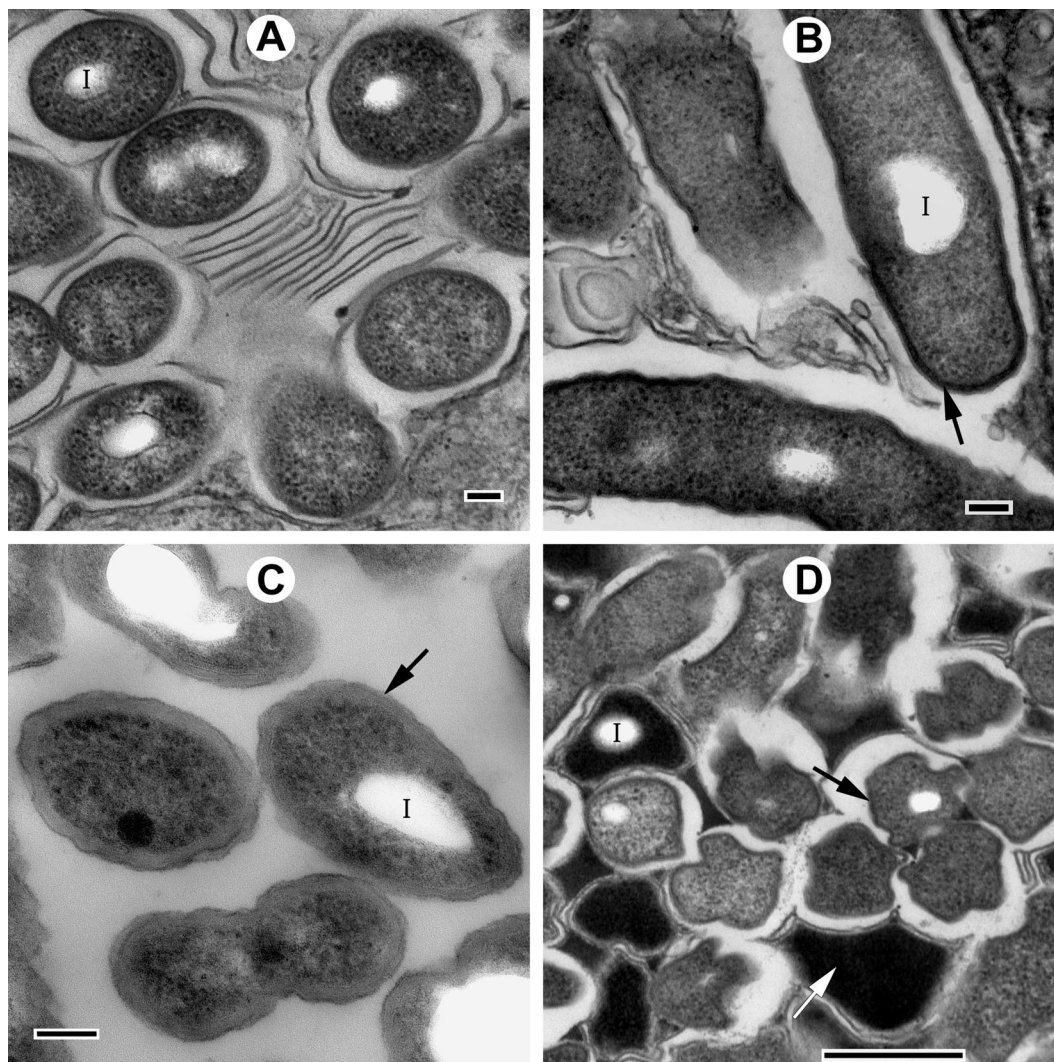


FIG. 2. *L. pneumophila* cells depicting typical characteristics of MIFs, in both mid-stage food vacuoles of *T. tropicalis* (A and B) and in expelled pellets (C and D). (A) Lp1-SVir cells depicting smooth (nonwavy) dark outer membranes, inclusions (I), and no apparent periplasm. (B) Lp02 cells showing an electron-dense thick layer in the inner leaflet of the outer membrane (arrow), the apparent lack of an inner membrane and periplasm, and inclusions (I). (C and D) Lp02 cells in pellets fixed 24 h postinoculation displaying thick laminated envelopes formed by the stacking of multiple layers of internal membranes (arrow in panel C), inclusions (I), electron-dense cytoplasm (white arrow in panel D), and irregular shapes (arrows in panel D). Bars, 100 nm (A to C) or 500 nm (D).

that food vacuoles and expelled pellets produced from the genetically complemented mutants still contained abundant outer membrane remains, with 10 to 20% of the complemented cells lacking multilayered envelopes, inclusions, an electron-dense cytoplasm, and/or irregular shapes.

Extended observations of *rpoS* and *letA* mutants in HeLa cells. To assess the ability of *rpoS* and *letA* mutants to form MIFs in other cellular models, and perhaps avoid the severe in vacuole degradation observed in *T. tropicalis*, HeLa cells (in which MIFs were first characterized [15, 23, 21]) were used. The two regulatory mutants were able to infect HeLa cells and proliferate, suggesting that their genetic defect is not growth limiting in mammalian cells. The *rpoS* mutant produced morphological forms characterized by a wavy outer membrane with sharp ripples and an electron-dense cytoplasm but lacking a multilayered envelope and irregular shapes (Fig. 5A and B).

Late in the infection process (~72 h), *rpoS* mutants associated with dead HeLa cells depicted a heterogeneous ultrastructure that included morphological characteristics typical of MIFs, i.e., nonwavy outer membranes and inclusions (Fig. 5B, inset). However, in ~90% of the latter mutants a periplasmic space was still distinguishable, and the inclusions were not as numerous as in Lp02 MIFs. In other words, only ~10% of the *rpoS* mutants found associated with dead HeLa cells showed the combined characteristics of MIFs, indicating that in HeLa cells the sigma factor RpoS is required for the full differentiation of *L. pneumophila* into MIFs. The *letA* mutant appeared even less able than the *rpoS* mutant to mature into MIFs or kill the host cell and remained in replicative vacuoles for extended periods (Fig. 5C). More than 95% of the *letA* mutants clearly showed the two membranes and periplasmic space typical of gram-negative envelopes and prominently depicted a wavy outer

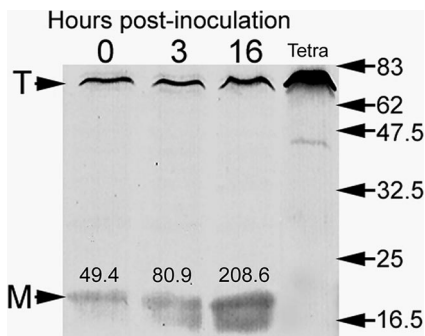


FIG. 3. Immunoblot showing that expression of MagA (a protein marker of MIF development in Philadelphia-1 strains) is upregulated during the interaction of Lp1-SVir with *T. tropicalis*. Samples were loaded based on CFU counts (5×10^4 /lane). The mean CFU/ml values of the two independent samples pooled at each time point were $6.3 \pm 1.52 \times 10^5$ (time zero), $8.5 \pm 1.95 \times 10^5$ (3 h), and $18.3 \pm 6.00 \times 10^5$ (16 h). The T arrowhead points to a *Tetrahymena* protein band recognized by the MagA polyclonal antibody. This protein band is well-labeled in samples containing ciliates only (lane labeled Tetra). The M arrowhead points at the position of MagA. Densitometry values in arbitrary IOD units are shown above each MagA band. The positions and molecular weights ($\times 1,000$) of broad-range, prestained protein standards are indicated on the right side.

membrane with sharp ripples, but inclusion bodies were not found (Fig. 5D). Therefore, in the HeLa cell infection model the *letA* mutants did not differentiate into MIFs, suggesting that the response regulator LetA is essential for the maturation of RFs into MIFs.

We have previously determined that SPFs and MIFs show Giménez-positive (red) staining, with MIFs in particular projecting a bright red color (21). In spite of the incomplete ultrastructural differentiation of the *rpoS* and *letA* mutants in HeLa cells, both mutants showed bright Giménez staining (not shown).

dot mutants morphologically differentiate into MIFs. We have previously shown that *L. pneumophila dot* mutants are largely digested in *T. tropicalis* food vacuoles (9). However, it is possible to find some vacuoles and expelled pellets containing undigested *dot* mutants. Thus, by increasing the sample size of transmission electron microscopy sections observed, we were able to establish that *dot* mutants displayed morphological features (Fig. 6) similar to those shown in Fig. 2 for the parent strain Lp02 MIFs. This observation indicated that the rapid morphological differentiation of SPFs into MIFs did not require a functional Dot/Icm system.

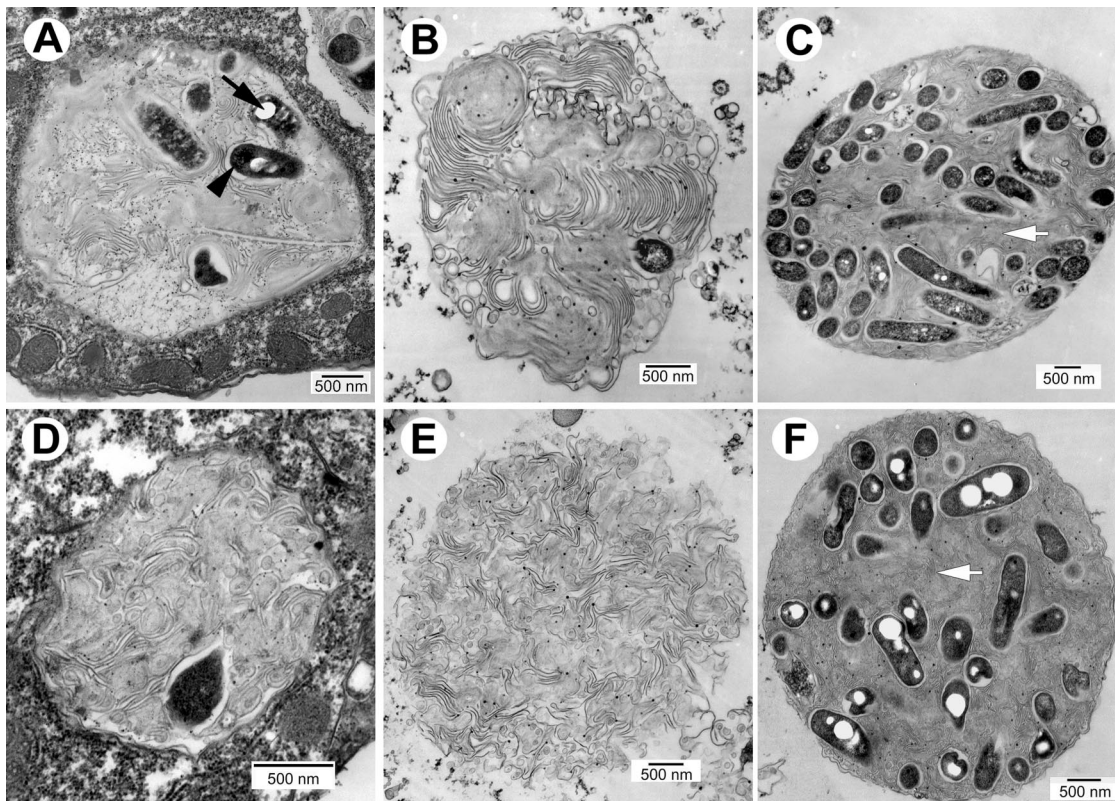


FIG. 4. Mutants unable to differentiate in vitro are readily digested by *T. tropicalis*. In two separate experiments Lp02 derivatives MB445 (*rpoS* mutant) (A and B) and MB434 (*letA* mutant) (D and E) did not survive in *T. tropicalis* vacuoles (A and D) and produced membranous pellets with virtually no surviving cells (B and E). Due to the very low number of morphologically intact bacteria it was not possible to assess bacterial morphology in a quantitative manner, but RpoS mutant survivors produced inclusions (arrow in panel A) and/or developed a smooth (nonwavy) outer membrane and dark cytoplasm (arrowhead in panel A), which are characteristics of MIFs. Genetically complemented *rpoS* and *letA* mutants recovered their ability to survive in food vacuoles and differentiate into MIFs. In expelled pellets from two separate samplings, the complemented *rpoS* and *letA* mutants (C and F, respectively) showed inclusions, straight dark envelopes, lack of an apparent periplasm, a dark cytoplasm, and irregular shapes. MIF-laden pellets in panels C and F still contained abundant membranous material in the space between bacterial cells (white arrows), suggesting that resistance to digestion did not reach parent strain (Lp02) levels.

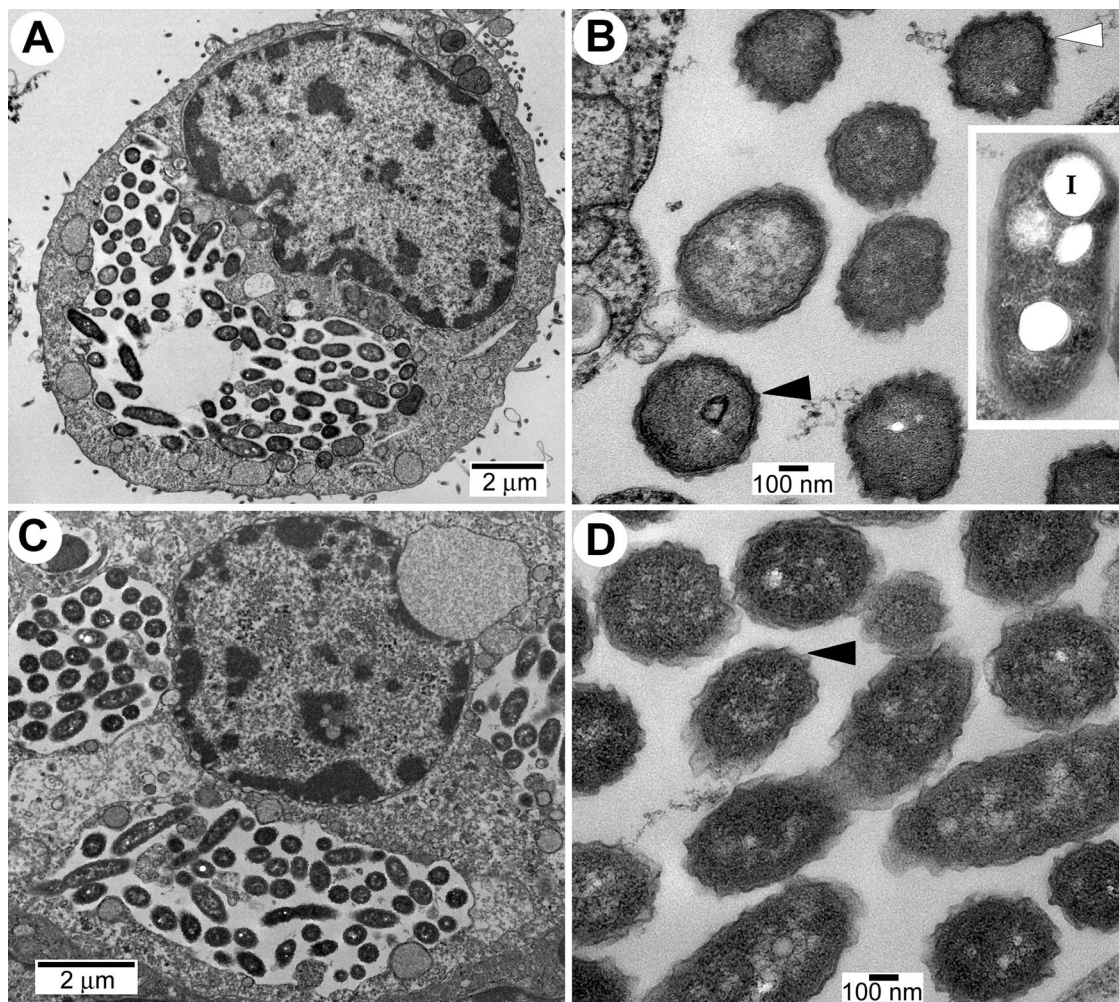


FIG. 5. Ultrastructure of *rpoS* and *letA* mutants in HeLa cells. *Legionella*-containing vacuoles of Lp02 derivatives MB445 ($\Delta rpoS$) (A and B) and MB434 (*letA* mutant) (C and D) depict bacterial cells with morphological characteristics similar to previously described intermediate developmental forms of *L. pneumophila* (15). (A and C) Low-magnification micrographs showing that the mutants largely remained inside replicative vacuoles in heavily infected, but morphologically intact, host cells even after 3 days postinfection, a time at which the parent strain Lp02 had already killed most infected cells and produced abundant MIFs. (B) In morphologically intact HeLa cells, >90% of the *rpoS* mutant cells had outer membranes with wavy outlines (black arrowhead) and sharp ripples (white arrowhead), but thick layered envelopes were typically absent. In those HeLa cells showing signs of cell death or lysis, the *rpoS* mutant showed a heterogeneous morphology, which included the above-described morphology (25%), bacteria with inclusions (70%), and those with inclusions (I) and straight outer membranes (35%, inset). (D) Cells of the *letA* mutant (>95%) presented a single morphology characterized by wavy outer membranes depicted by sharp ripples (arrowhead) and did not develop inclusions. At least 50 infected cells were looked at in two separate samplings (for a total of ~100 infected cells observed) of ultrathin sections cut from a single block.

DISCUSSION

The developmental biology of *L. pneumophila* is beginning to be examined in molecular detail, and the study of SPFs has been crucial for the elucidation of regulatory networks that control the differentiation of *L. pneumophila* in vitro (reviewed in references 19 and 35). However, in vivo (i.e., intracellular differentiation) progress has been limited by the lack of experimental models where the complete and synchronous differentiation of *L. pneumophila* into MIFs can be achieved. Here we report that the ciliate *T. tropicalis* constitutes a useful experimental model to both study MIF development in vivo and explore the developmental relationships that may exist between SPFs and MIFs. By showing that SPFs directly and rapidly differentiate into MIFs while in transit through *T. tropi-*

calis, we have now proven that SPFs represent a stable intermediate in the differentiation of RFs to MIFs and have addressed previously unanswered questions in relation to the mechanism of *L. pneumophila* differentiation in vitro versus in vivo.

MIF development in *T. tropicalis* was recorded as early as 30 min after the addition of the bacterial inoculum. This rapid and direct in vacuole differentiation of SPFs into MIFs stands in sharp contrast to the differentiation of RFs into MIFs in HeLa cells (15, 23) and amoebae (24), a process preceded by bacterial replication that thus appears to be lengthy. In fact, MIF development may be equally fast in *T. tropicalis*, HeLa cells, and amoebae, once the right conditions for differentiation are met. In macrophages derived from the U937 human monocytic

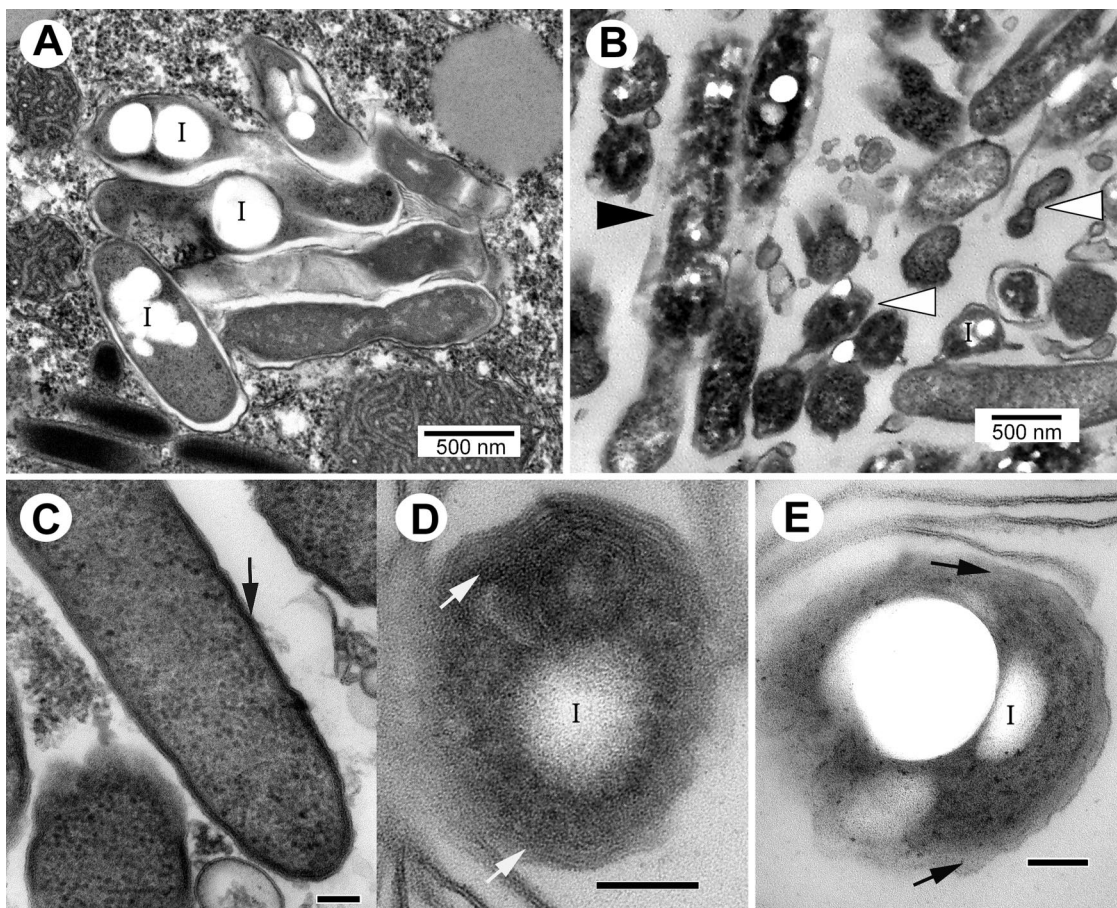


FIG. 6. Differentiation of *dot* mutant SPFs into MIFs as observed by electron microscopy in *T. tropicalis* food vacuoles (A and B) or expelled pellets (C to E). (A and C) *dotA* mutant; (B, D, and E) *dotB* mutant. (A and B) Early development of MIFs was evident in mid-stage food vacuoles for both mutants. While several bacteria appeared degraded (e.g., black arrowhead in panel B), small groups of bacteria could be seen with irregular shapes, dark cytoplasm, and thick envelopes (white arrowheads in panel B), straight outer membranes with no evidence of periplasm, and inclusions (I). (C to E) In pellets, structurally complete bacteria also exhibited features typical of MIFs, e.g., thick, dark, and nonwavy outer membrane (arrow in panel C), multiple internal membranes (arrows in panel D), a laminated envelope with no obvious periplasm (arrows in panel E), dense cytoplasm, and prominent inclusions (I). Bars in panels C to E, 100 nm.

cell line, *L. pneumophila* does not fully develop into MIFs (21). We have thus hypothesized that the early apoptotic demise of macrophages results in insufficient time for differentiation to take place (21). In light of the results presented here, this hypothesis may require revision in favor of a possible failure in molecular signaling inside macrophages. Whereas in HeLa cells and amoebae the signals that trigger differentiation are seemingly delivered late in the growth cycle (after bacterial replication), in human macrophages these signals may not be present. The study of *L. pneumophila* gene expression in response to the vacuolar environment of *Tetrahymena*, as well as the characterization of such an *in vivo* environment, may present a rare opportunity to identify the signals that drive the direct differentiation of SPFs into MIFs.

Using the model presented in Fig. 7, we can now speculate about the nature of the signal(s) that triggers MIF development in *T. tropicalis*. Legionellae-laden food vacuoles always contain varied amounts of outer membrane remains (9) (Fig. 1), suggesting that the contained legionellae are exposed to *T. tropicalis* digestive factors. The rapid differentiation of SPFs

into MIFs, the latter being characterized as the most resilient form of *L. pneumophila* (1, 6, 7, 21), could be viewed as an attempt by *L. pneumophila* to enhance survival in the hostile environment of the *Tetrahymena* food vacuole. If this were the case, the signals that trigger *L. pneumophila* differentiation might include structural damage to the bacterial cell envelope or the sensing of specific digestive enzymes. It is possible that this type of signal is also delivered in amoeba, particularly if the amoebal *Legionella*-containing vacuole fuses with lysosomes and becomes acidic late in the growth cycle, as reported to occur in bone marrow-derived mouse macrophages (41). Moreover, because the late *Legionella*-containing vacuole is not acidified in human macrophages (44), the signal would be incomplete in these cells, potentially explaining why MIFs do not fully develop in U937-derived macrophages. Alternatively, a host factor secreted into the food vacuole by the ciliate, or actively imported into the food vacuole by *L. pneumophila*, may constitute the signal that, in ciliates, triggers MIF development.

One requirement to be met by the signals that trigger the

in culture) may be a factor to consider in the lack of person-to-person transmission of Legionnaires' disease.

Finally, the *T. tropicalis* infection model also offered the opportunity to separate (unlink) the processes of *L. pneumophila* differentiation and intracellular replication. Previously, in all the cellular models of differentiation, a replication phase was required before MIFs could be formed. Therefore, it had been impossible to determine whether *dot/icm* mutants failed to produce MIFs in vivo (in spite of being capable of differentiating into SPFs in vitro) as a consequence of their inability to replicate intracellularly, or because MIF development requires a functional Dot/Icm system. Using the *Tetrahymena* differentiation model we have now determined that mutants with a defective Dot/Icm virulence system morphologically differentiate into MIFs. In summary, *T. tropicalis* has allowed us to unequivocally conclude that SPFs directly differentiate into MIFs, and that the Dot/Icm system is not required for the maturation of *L. pneumophila* into MIFs, two events that could not be experimentally addressed before. The *Tetrahymena* model could now be exploited to study the signals that trigger differentiation into MIFs and as the only replication-independent model reported to date that allows the maturation of Dot/Icm mutants and perhaps other intracellular growth-deficient mutants of *L. pneumophila*.

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