**Legionella pneumophila Replication Vacuoles Mature into Acidic, Endocytic Organelles**

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**Abstract**

After ingestion by macrophages, *Legionella pneumophila* inhibits acidification and maturation of its phagosome. After a 6–10-h lag period, the bacteria replicate for 10–14 h until macrophage lysis releases dozens of progeny. To examine whether the growth phase of intracellular *L. pneumophila* determines the fate of its phagosome, interactions between the endosomal network and pathogen vacuoles were analyzed throughout the primary infection period. Surprisingly, as *L. pneumophila* replicated exponentially, a significant proportion of the vacuoles acquired lysosomal characteristics. By 18 h, 70% contained lysosomal-associated membrane protein 1 (LAMP-1) and 40% contained cathepsin D; 50% of the vacuoles could be labeled by endocytosis, and the pH of this population of vacuoles averaged 5.6. Moreover, *L. pneumophila* appeared to survive and replicate within lysosomal compartments: vacuoles harboring more than five bacteria also contained LAMP-1, inhibition of vacuole acidification and maturation by bafilomycin A1 inhibited bacterial replication, bacteria within endosomal vacuoles responded to a metabolic inducer by expressing a *gfp* reporter gene, and replicating bacteria obtained from macrophages, but not broth, were acid resistant. Understanding how *L. pneumophila* first evades and then exploits the endosomal pathway to replicate within macrophages may reveal the mechanisms governing phagosome maturation, a process also manipulated by *Mycobacteria*, *Leishmania*, and *Coxiella*.

Key words: pathogenesis • autophagy • phagosomes • lysosomes • macrophages

**Introduction**

After inhalation, the gram-negative bacillus *Legionella pneumophila* can replicate within alveolar macrophages and cause severe pneumonia in immunocompromised people. The reservoir for *L. pneumophila* appears to be freshwater amebae, which also phagocytose but do not digest this opportunistic pathogen. Thus, *L. pneumophila* surmounts the formidable antimicrobial activities of professional phagocytes and establishes an intracellular niche that provides a ready supply of nutrients, protection from environmental stresses, and freedom from competition with other microbes.

Phagosomes harboring *L. pneumophila* have several unique features. After their internalization by coiling phagocytosis (1), the bacteria persist for at least 8 h in phagosomes that neither acidify nor fuse with lysosomes (2–7). Instead, by 4 h, endoplasmic reticulum envelopes the vacuole, a process that resembles autophagy (3, 8). After a lag phase of 6–10 h, bacterial replication begins. By 24 h, the number of *L. pneumophila* has increased 50–100-fold, and lysis of phagocytes is evident (3, 9, 10). Several factors required by *L. pneumophila* to establish its protective vacuole have been discovered (11), but their modes of action remain elusive.

Broth cultures of *L. pneumophila* coordinately express several virulence traits in response to growth conditions (12). When amino acids are limiting, the second messenger ppGpp accumulates and triggers expression of traits likely to promote bacterial transmission to another phagocyte (13). Accordingly, postexponential phase (PE)¹ *L. pneumophila* are competent to evade phagosome–lysosome fusion, whereas 90% of exponential phase (E) bacteria are destroyed rapidly in macrophage lysosomes. Whether *L. pneumophila* also downregulates competence to evade lysosomes during growth in macrophages has not been established, as few studies have analyzed phagosomes >8 h old.

¹Abbreviations used in this paper: BFA, bafilomycin A1; E, exponential phase; GFP, green fluorescent protein; IPTG, isopropyl-thio-β-D-galactopyranoside; LAMP-1, lysosomal-associated membrane protein 1; MOI, multiplicity of infection; PE, postexponential phase; TR-DEX, Texas red–dextran; TR-OV, Texas red–ovalbumin.

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The growth phase of an intracellular pathogen can affect phagosome maturation. Infectious *Leishmania* promastigotes evade phagosome–lysosome fusion (14), but, concomitant with their transformation into replicative amastigotes, the phagosomes merge with lysosomes, wherein the protozoa replicate (15–17). Similarly, the dormant small-cell variant form of *Coxiella burnetii* delays phagosome maturation (18); subsequently, its large-cell variant form replicates within phagolysosomes (19–21). Therefore, we tested the hypothesis that the growth phase of intracellular *L. pneumophila* determines the fate of its vacuole.

**Materials and Methods**

**Bacteria Cultures.** *L. pneumophila* Lp02, a thymine auxotroph derived from Philadelphia strain 1 (22), was cultured in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma-Aldrich) buffered yeast extract broth containing 135 μg/ml ferric nitrate, 400 μg/ml L-cysteine, and 100 μg/ml thymidine (AYET). Macrophages were infected with *L. pneumophila* cultured to PE as assayed by absorbance at 600 nm of OD 3.3–4.0 and motility of >20% of the cells; E cultures were of OD 0.8–1.2 and nonmotile (12). Positive controls for phagosome–lysosome maturation assays were PE *L. pneumophila* incubated for 20 min at 80°C, which reduced viability 99.9%, and PE *Escherichia coli* strain DH5α cultured in Luria-Bertani broth and labeled with 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (NHS-carboxyfluorescein; Roche) as described for pH assays. For some assays, labeled *E. coli* were fixed in 2.5% formaldehyde/PBS for 0.5 h and washed three times with PBS.

**Macrophage Cultures.** Cultures of bone marrow–derived macrophages from A/J mice (The Jackson Laboratory) were prepared as described previously (8).

**Microscopy.** 2 × 10⁵ macrophages per 12-mm glass coverslip were infected at 37°C for 1 h with *L. pneumophila* at a multiplicity of infection (MOI) of 0.5 in RPMI 1640 containing 10% heat-inactivated FCS (RPMI/FCS), washed three times in medium, then incubated for the period indicated in RPMI/FCS containing 100 μg/ml thymidine. To analyze the role of endosomal acidification, macrophages were infected for 1 h with *L. pneumophila*, washed three times in RPMI/FCS, then incubated for the times indicated in medium containing 25 mM bafilomycin A1 (BFA; Calbiochem). Cells were fixed for 0.5 h in periodate-lysine-paraformaldehyde containing 5% sucrose (8), washed once in PBS containing 5% sucrose, then permeabilized for 1 min with 1 mg/ml Zwittergent (Roche) per ml PBS. To reduce nonspecific binding of antibodies, PBS containing 5% sucrose and 2% goat serum was used to preincubate cells and for antibody dilutions, as follows: rabbit anti–*L. pneumophila* (a gift from Dr. Ralph Isberg, Howard Hughes Medical Institute and Tufts University School of Medicine, Boston, MA), 1:1,000; rat anti–lysosomal-associated membrane protein 1 (LAMP-1) (ID4B; Developmental Hybridoma Bank), 1:100; rabbit anti–cathepsin D (a gift from Dr. Sadaki Yokota, Yamashini Medical University, Yamashini, Japan), 1:100; rabbit anti–procathepsin D (23), 1:20; and rat anti–βII (a gift of David Bole, University of Michigan, Ann Arbor, MI), 1:200; all fluorescent secondary antibodies (Molecular Probes) were diluted 1:2,000. Cells were incubated with antibodies for 1 h at 37°C, then washed three times in PBS containing 5% sucrose. *L. pneumophila* were stained with either anti–*L. pneumophila* antibody or 0.1 μM of the nucleic acid dye 4′,6′-diamino-2-phenylindole (DAPI; Molecular Probes).

Samples were analyzed with a ZEISS Axioplan 2 epifluorescence microscope equipped with a 100X Plan-Neofluar objective, numerical aperture of 1.3, and filters 487901, 487910, and 487900. 50 vacuoles containing *L. pneumophila* were scored per coverslip, and no more than three vacuoles per cell were counted. Vacuoles were scored as positive for soluble markers when any fluorescence was detected within their lumens. For membrane markers, vacuoles were defined as positive only when a complete ring of fluorescence was observed at the vacuole periphery; this stringent criterion likely underestimates the fraction of LAMP-1–containing vacuoles. As >20 bacteria per vacuole could not be counted accurately, these were scored as >20. However, their size indicated that mature vacuoles held >50 bacteria.

**Access to Endocytosed Material.** To label lysosomes fluorescently, macrophages were cultured for 0.5 h with 100 μg Texas red–ovalbumin (TR-OV) or Texas red–dextran (TR-DEX), 10 kD (Molecular Probes) per ml of RPMI/FCS, washed, incubated for 0.5 h in unlabeled medium, then infected with *L. pneumophila* at an MOI of 0.5. For pulse–chase experiments, infected macrophages were incubated with 250 μg/ml TR-OV for 15 min, unlabeled RPMI/FCS for an additional 5–120 min, then were fixed, permeabilized, stained with DAPI, and scored as described above.

**pH Measurement.** To determine the pH of phagosomes <6 h old, bacteria were first surface labeled with a ratiometric fluorophore. Either *L. pneumophila* or *E. coli* were washed once in PBS, labeled with 1 mg NHS-carboxyfluorescein per ml of PBS, pH 8, for 0.5 h at 4°C, then washed three times in RPMI/FCS. Macrophages cultured on 25-mm coverslips were infected with *L. pneumophila* for 3–6 h at an MOI of 0.5 and with *E. coli* for 1–2 h at an MOI of 2. Surface labeling did not affect bacteria viability, as determined by colony formation, and only modestly affected *L. pneumophila* trafficking: 2 h after infection, an average of 20% labeled bacteria fused with lysosomes compared with 10% of control bacteria.

The pH of those pathogen vacuoles that were accessible to the endosomal pathway was also measured. Macrophages were infected with *L. pneumophila* at an MOI of 0.5 for 1 h, washed, then incubated for the period specified. 2 h before each end point, macrophages were incubated for 0.5 h with fluorescein dextran, 10 kD (Molecular Probes) at 200 μg/ml, then for 1.5 h in unlabeled RPMI/FCS. To test whether macrophage proton ATPase activity affected vacuolar pH, macrophages were treated with 1 μM BFA for 1 h before the end point. To label intracellular bacteria, DAPI was added to 0.3 μM for 30–45 min before the end point. DAPI did not affect vacuolar pH: the pH of phagosomes containing *L. pneumophila* surface labeled with fluorescein was similar to the pH of those harboring bacteria labeled with both fluorescein and DAPI.

Vacular pH was measured by the method of Tsang et al. (24) using Metamorph software (Universal Imaging Corp.). Infected macrophages were washed twice with 37°C Ringers buffer (155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM Na₂HPO₄, 10 mM Heps, and 10 mM glucose, pH 7.2), mounted into a 37°C coverslip holder, and visualized on a Nikon eclipse TE300 microscope with an attached Quantix Photometrics camera (Princeton Instruments). Vacuoles containing *L. pneumophila* were identified by DAPI fluorescence at 405-nm excitation (450-nm emission), then images of pathogen vacuoles that had acquired dextran were collected at 440- and 485-nm excitation (520-nm emission). The DAPI image was masked to define the bacterial pixel area, and the average gray area of the corresponding pixels in the 440- and 485-nm images was recorded.
The pH of each vacuole was calculated as the ratio of 485-nm intensity (pH sensitive) to 440-nm intensity (pH insensitive). Calibration for individual fluorescent particles was conducted in situ for each experiment using cells treated with nigericin and isotonic potassium buffers of defined pH as described by Beauregard et al. (25). Young phagosomes were analyzed by masking the 440-nm image, then the ratios were calculated as described above. As a control, the lysosomes of uninfected macrophages were labeled with fluorescein dextran as described above, the 440-nm image was masked, then ratios for all of the vesicles in the field were calculated as described above.

Viability of Intracellular L. pneumophila. Plasmid pMMB-GRN was constructed from which expression of green fluorescent protein (GFP) by L. pneumophila could be induced by isopropyl-thio-
β-D-galactopyranoside (IPTG; Sigma). An SmaI–HindIII fragment from pGFPmut3 (26) encoding GFP was ligated 3' to the Pm promoter of pMMB-Gent, which confers gentamicin resistance (13). To eliminate their inhibitory effect on L. pneumophila replication (27), mobA and mobB were deleted by digestion of pMMB-GRN with AgeI followed by intramolecular ligation. pMMB-GRN was transferred to L. pneumophila by electroporation and selection on CYET plates containing 10 μg/ml gentamicin. Analysis of both cultures indicated GFP expression could be detected after an IPTG treatment of 4 h, or approximately two generation periods (data not shown).

To evaluate GFP expression, infected macrophages were incubated in 2 mM IPTG for 1.5 h, in TR-100 and IPTG for 0.5 h, then in unlabeled RPMI/FCS containing IPTG for 2 h. After fixation and DAPI staining, vacuoles were scored both for endosomal TR-100 and GFP fluorescence. Alternatively, macrophages were infected for 1 h, washed, incubated for an additional 13–17 h, treated with 2 mM IPTG for 4 h, then their LAMP-1 was stained as described above.

Intracellular Growth. 105 macrophages per well of 48-well tissue culture dishes were infected for 1 h with L. pneumophila at an MOI of 0.5, washed three times with RPMI/FCS, then incubated for 2–17 h. Macrophages were lysed with 300 μl of PBS/saponin adjusted with HCl to either pH 6.9 or 5.0, then the lysis volume was brought to 1.5 ml with AYET of either pH 6.9 or 5.0. In parallel, E or PE L. pneumophila cultures in pH 6.9 AYE were diluted to 2 × 106 cells per ml of AYET of either pH 6.9 or 5.0 and containing 300 μl of either PBS/saponin or lysate prepared from 1.5 × 108 uninfected macrophages. After incubation at 37°C on a rotating wheel for 1–4 h, aliquots of four 10-fold dilutions of each sample were cultured for 3–4 d on CYET. Survival was calculated as (CFU/ml AYET, pH 5.0)/(CFU/ml AYET, pH 6.9) × 100.

Results

Mature L. pneumophila Vacuoles Interact with the Lysosomal Compartment. To determine whether mature L. pneumophila vacuoles acquire characteristics of late endosomes and lysosomes, infected macrophages were analyzed by fluorescence microscopy after labeling with antibodies specific for the LAMP-1 or cathepsin D proteins, two hallmarks of the late stages of phagolysosome development (28–30), or soluble fluorescent endosomal tracers.

As expected, few early L. pneumophila vacuoles acquired the late endosomal and lysosomal protein LAMP-1 (6, 7; Fig. 1, A and B). However, by 16 h, 70% of the vacuoles contained this glycoprotein. LAMP-1–specific fluorescence was associated with the periphery of L. pneumophila vacuoles, suggesting either localization within the vacuolar membrane or juxtaposition of small vesicles to the vacuole membrane. In many cells, the lysosomes had coalesced around the bacterial vacuole (24 h; Fig. 1 A).

The lysosomal protease cathepsin D also accumulated in mature L. pneumophila vacuoles. Early in infection, <20% of the L. pneumophila phagosomes contained this enzyme, as reported previously (5); by 16 h, 40% or more of the vacuoles did so (Fig. 1, A and C). As expected, this soluble enzyme appeared to be in the lumen of L. pneumophila vacuoles. In contrast, the proform of cathepsin D, which is delivered by the biosynthetic pathway to early endosomes (31, 32), was not detected within mature L. pneumophila vacuoles (data not shown).

A similar result was obtained when macrophages whose lysosomes were prelabeled with the soluble fluorescent tracers TR-100 or TR-DEX were infected with L. pneumophila. Within 5 h of infection, <20% of the vacuoles contained the fluorescent probes; however, by 18–20 h, at least 50% had fused with lysosomes (Fig. 2). Next, the accessibility of L. pneumophila vacuoles to the endocytic pathway was assessed. Macrophages that had ingested either formaldehyde-fixed E. coli or live L. pneumophila were allowed to pinocytose TR-100 for 15 min, then the cells were incubated in fresh medium for an additional 5, 30, or 120 min. As expected, after the 120-min chase, ~80% of phagosomes containing E. coli had accumulated fluid phase probe, whereas <20% of the nascent L. pneumophila vacuoles had done so (Fig. 3). In

Acid Tolerance. 1.5 × 106 macrophages per well of a 6-well tissue culture dish were infected for 1 h with L. pneumophila at an MOI of 0.5, washed three times with RPMI/FCS, then incubated for 2–17 h. Macrophages were lysed with 300 μl of PBS/saponin adjusted with HCl to either pH 6.9 or 5.0, then the lysis volume was brought to 1.5 ml with AYET of either pH 6.9 or 5.0. In parallel, E or PE L. pneumophila cultures in pH 6.9 AYE were diluted to 2 × 106 cells per ml of AYET of either pH 6.9 or 5.0 and containing 300 μl of either PBS/saponin or lysate prepared from 1.5 × 108 uninfected macrophages. After incubation at 37°C on a rotating wheel for 1–4 h, aliquots of four 10-fold dilutions of each sample were cultured for 3–4 d on CYET. Survival was calculated as (CFU/ml AYET, pH 5.0)/(CFU/ml AYET, pH 6.9) × 100.

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contrast, 50% of the mature pathogen vacuoles contained TR-OV.

Although a significant proportion of mature replication vacuoles accumulated detectable quantities of LAMP-1, cathepsin D, and the endosomal tracers TR-OV and TR-DEX, not all of the vacuoles did so. However, analysis of mature replication vacuoles was hampered by two technical limitations. First, as vacuoles can be scored as positive only
after accumulating visible quantities of marker, the earliest interactions with the endosomal pathway could not be detected. Second, by viewing live cultures by phase microscopy, it was evident that macrophages containing large replication vacuoles had rounded and many had detached from the coverslip. As this population of cells would be washed away during the immunofluorescence staining procedure, mature replication vacuoles likely are underrepresented in our data sets. Indeed, when macrophages were cultured on tissue culture-grade coverslips (no. 12-545-82; Fisher Scientific), 61% of mature replication vacuoles contained detectable TR-OV (50 vacuoles scored in each of three experiments). Taken together, the apparent absence of procathepsin D and the presence of LAMP-1, cathepsin D, and the endosomal tracers TR-OV and TR-DEX in a significant fraction of bacterial phagosomes indicated that as they age, *L. pneumophila* vacuoles merge with the lysosomal compartment.

**Endosomal *L. pneumophila* Vacuoles Are Acidic.** To evaluate *L. pneumophila* phagosome maturation by an independent approach, we compared their pH to that of conventional phagolysosomes. Similar to the results obtained by Horwitz and Maxfield (2), phagosomes aged 3–6 h that contained live NHS-carboxyfluorescein–labeled *L. pneumophila* maintained a neutral pH (average pH 7.4), whereas vacuoles harboring similarly labeled *E. coli* or heat-killed *L. pneumophila* exhibited acidic pH values of 5.5 and 5.6, respectively (Table I). Next, the pH of those mature *L. pneumophila* vacuoles that had merged with the endosomal pathway was measured. For this purpose, infected macrophages were allowed to endocytose fluorescein-dextran, a process that labels nearly 50% of the mature *L. pneumophila* vacuoles (Fig. 2); the remaining bacterial vacuoles could not be evaluated. By 16–20 h after infection, the majority of fluorescein-dextran–containing *L. pneumophila* vacuoles were acidic, with an average pH of ~5.6, a value comparable to that of lysosomal vesicles in uninfected macrophages (Table I).

Lysosomes are acidified by the proton ATPase (33). To determine whether acidification of mature *L. pneumophila* vacuoles required the proton ATPase, infected macrophages were incubated with a specific inhibitor of its activity, BFA (34). After a 1-h treatment, mature *L. pneumophila* vacuoles were pH 7.3; likewise, lysosomal vesicles in uninfected macrophages were pH 7.4 after BFA treatment.

### Table I. pH of Young and Mature Vacuoles

| Condition                        | Age       | n*  | pH  | Error |
|---------------------------------|-----------|-----|-----|-------|
| Phagosomes containing           |           |     |     |       |
| NHS-carboxyfluorescein–labeled  |           |     |     |       |
| particles†                      | Live *E. coli* | 45–75 min | 17 | 5.6  | 0.32 |
|                                 | Live *L. pneumophila* | 3–6 h | 56 | 7.4  | 0.9  |
|                                 | Heat-killed *L. pneumophila* | 2–4 h | 41 | 5.5  | 0.3  |

| Vacuoles containing             |           |     |     |       |
| fluorescent dextran§            |           |     |     |       |
| *L. pneumophila*                | 16–22 h   | 64  | 5.6 | 0.8  |
| *L. pneumophila* + 1 µM BFA¶    | 17–20 h   | 22  | 7.3 | 0.8  |
| Macrophage vesicles§            | 2 h       | 300 | 5.5 | 0.3  |
| Macrophage vesicles + 1 µM BFA | 2 h       | 90  | 7.6 | 0.59 |

*Data represent one (*E. coli*), four (live *L. pneumophila*), or three (heat-killed *L. pneumophila*) independent experiments, or data represent five (*L. pneumophila*) or two (*L. pneumophila* + BFA, vesicles, and vesicles + BFA) independent experiments.

+Bacteria were surface labeled with carboxyfluorescein before infection as described in Materials and Methods.

+To label the endosomal network and pathogen vacuoles, infected or uninfected macrophages were incubated for 2 h with fluorescein dextran as described in Materials and Methods.

+To determine the effect of proton ATPase activity on the pH of pathogen vacuoles and endosomal vesicles, infected or uninfected macrophages were treated with BFA for 1 h before ratiometric image analysis as described in Materials and Methods.

+Endosomal vesicles of uninfected macrophages were analyzed as described in Materials and Methods.
(Table I). Therefore, macrophage proton ATPase activity was required to acidify mature L. pneumophila vacuoles.

**L. pneumophila Survival and Replication within Endocytic, Acidic Vacuoles.** The observation that a significant proportion of mature L. pneumophila vacuoles acidify, accumulate endocytic probes, and contain LAMP-1 and cathepsin D strongly suggested that L. pneumophila are delivered to a lysosomal compartment during the intracellular replication period. Therefore, the viability and replication of L. pneumophila within lysosomal vacuoles was assessed by four methods.

First, at 8, 16, and 24 h after infection, the number of bacteria residing in each nonendosomal vacuole was compared with that in each endosomal vacuole, as judged by the presence of LAMP-1. The majority of vacuoles that harbored more than five bacteria were LAMP-1 positive, and the bacterial density within these endosomal vacuoles increased with time (Fig. 4 A). In contrast, vacuoles that lacked LAMP-1 typically contained less than five bacteria. A minority of vacuoles contained >20 bacteria yet lacked detectable LAMP-1, indicating that bacterial replication can precede accumulation of endosomal markers. We also observed that even 16 and 24 h after infection, approximately half of the bacterial vacuoles contained less than five bacteria. Although macrophages were infected with PE broth cultures, intracellular bacterial replication was not synchronous (Fig. 4); thus, this vacuole population likely represents both cells that never replicated during the primary infection and secondary infections by PE bacteria. Furthermore, because cells containing large replication vacuoles detach from the coverslips, the actual fraction of infected macrophages that contain vacuoles bearing one to five bacteria is likely to be smaller than the calculated value. Thus, microscopic analysis of individual phagosomes (Fig. 4 A) indicated that interaction with the endosomal network did not inhibit, and perhaps promoted, L. pneumophila replication.

Next, the effect of neutralizing the macrophage endosomal pathway on L. pneumophila growth was examined. From 8 to 24 h of infection, the number of L. pneumophila in macrophage cultures increased ~20-fold (Fig. 5). However, in macrophages whose endosomal network was neutralized with BFA, the yield of L. pneumophila increased only twofold. Enumeration of the bacteria in each vacuole confirmed and extended these results. In untreated macrophages 24 h after infection, 26% of L. pneumophila vacuoles harbored >11 bacteria (Fig. 4 A). In contrast, in BFA-treated macrophages, only 8% of the vacuoles contained >11 bacteria (Fig. 4 B). Notably, neutralization of the endosomal compartment did not block bacterial replication at the single cell stage. Apparently, an acidic endosomal network is not a prerequisite for initiation of bacterial replication, but macrophage proton ATPase activity did promote intracellular growth of L. pneumophila.

Concomitant with its inhibition of L. pneumophila replication, BFA also retarded the acquisition of late endosomal and lysosomal proteins by pathogen vacuoles. In untreated macrophages, 50% or more of mature L. pneumophila vacuoles contained LAMP-1 and accumulated TR-OV (Fig. 2), whereas after BFA treatment only ~20% of the mature vacuoles did so (Fig. 6).

![Figure 4](image1.png)  
**Figure 4.** The bacterial density in vacuoles that contained LAMP-1 (black bars) or lacked LAMP-1 (white bars). (A) Macrophages infected for the period indicated were fixed and processed for microscopic localization of LAMP-1 and L. pneumophila. At the times indicated, vacuoles were scored for the presence of LAMP-1 and the number of bacteria. As it was not possible to count accurately >20 bacteria in one compartment, these were scored as >20. (B) After a 1-h infection, macropahges were treated with the proton ATPase inhibitor BFA, incubated for the times indicated, then processed as described above. In each of three independent experiments, 50 vacuoles were analyzed for each condition at the times indicated. The population of vacuoles analyzed for A was the same as that depicted in Fig. 1 B; the population of vacuoles analyzed for B was the same as that represented by Fig. 7.

![Figure 5](image2.png)  
**Figure 5.** The inhibition of L. pneumophila growth in macrophages by the proton ATPase inhibitor BFA. Infected macrophages were incubated with 25 nM BFA (open circles) or without drug (filled squares), and the yield of CFU was determined at the times indicated. Shown are the means and SE values calculated from four separate experiments, each performed in triplicate.
Although the interaction of the pathogen vacuole with the endocytic pathway appeared to be pH sensitive, its association with the endoplasmic reticulum was not. In control cultures, by 6 h after infection, 54% of the *L. pneumophila* vacuoles were enveloped by endoplasmic reticulum, as judged by immunofluorescence microscopic localization of BiP ($n = 50$ in each of three experiments [8]). Likewise, when infected macrophages were treated with BFA, 58% of pathogen vacuoles colocalized with BiP ($n = 50$ in each of three experiments). Furthermore, the yield of *L. pneumophila* CFU 24 h after infection was similar whether or not the cultures were treated with BFA during the first 6–8 h of infection (data not shown). Therefore, the pH-sensitive stage of the *L. pneumophila* life cycle occurs after formation of its autophagosome-like vacuole.

Results of several control experiments indicated that BFA did not affect bacterial or macrophage viability. First, growth of *L. pneumophila* in broth was not inhibited by even 500 nM BFA (data not shown). Second, BFA treatment did not permanently disrupt endosomal traffic. When macrophages were treated with BFA for 24 h before, rather than during, the infection with *L. pneumophila*, no difference in vacuole maturation or the yield of CFU was observed between pretreated and untreated cells (data not shown). Third, neutralization of the acidic compartments of cells, including phagosomes, with 25 μM chloroquine or 25 mM ammonium chloride (35, 36) also inhibited *L. pneumophila* replication, although to a lesser extent than did BFA (data not shown). Therefore, *L. pneumophila* growth was maximal within macrophages whose endosomal compartment was acidic and competent to fuse with the bacterial vacuole.

Third, to assess the metabolic activity of *L. pneumophila* residing within endocytic vacuoles, macrophages were infected with a strain in which GFP expression could be induced by IPTG. After 16 h, a time when >60% of the replication vacuoles have merged with the endosomal pathway (Fig. 1), macrophages were incubated with IPTG and TR-OV for 4 h, then the capacity of bacteria within lysosomal vacuoles to express GFP was analyzed microscopically. Approximately 70% of the pathogen vacuoles harbored bacteria that expressed GFP, whether or not the vacuole had accumulated either LAMP-1 or TR-OV (Fig. 7 and Table II). Therefore, *L. pneumophila* appeared to survive within lysosomal vacuoles.

Finally, the prediction that *L. pneumophila* replicating in macrophages are acid resistant was tested. Macrophages infected with *L. pneumophila* for 3, 12, or 18 h were lysed, and the bacteria were transferred to broth of pH 6.9, which is standard, or pH 5.0, to mimic the lysosomal compartment (Table I). In parallel, E and PE broth cultures were incubated in AYET of pH 6.9 or 5.0. As is typical of many bacteria (37), PE *L. pneumophila* were acid resistant, whereas acid treatment reduced by 1,000-fold the CFU of E *L. pneumophila* (Fig. 8). In striking contrast, a similar treatment of replicating bacteria obtained from macrophages reduced the CFU less than fivefold. Macrophage lysates did not induce acid tolerance of *L. pneumophila*, as...
broth cultures of E. L. pneumophila incubated with lysates prepared from uninfected macrophages were not protected from the pH 5.0 stress (data not shown). The prediction that L. pneumophila are replicating 12 and 18 h after infection (Figs. 1, 2, 4, and 5) was confirmed by the twofold increase in CFU that was observed after incubating parallel samples of macrophage lysates in AYET, pH 6.9 for 3 h, a period corresponding to less than two L. pneumophila doubling times (data not shown). Thus, consistent with our observation that a significant proportion of mature bacterial replication vacuoles are pH 5.6 (Table I), the phagosomal environment of macrophages appeared to induce L. pneumophila acid resistance.

### Table II. Comparison of GFP Expression by Bacteria within Endosomal Versus Nonendosomal Vacuoles

| Endosomal marker | GFP− | GFP+ | Total vacuoles* | Percent GFP+ |
|------------------|------|------|-----------------|--------------|
| LAMP-1 negative  | 28   | 66   | 94              | 70           |
| LAMP-1 positive  | 46   | 111  | 157             | 71           |
| TR-OV negative   | 75   | 187  | 262             | 71           |
| TR-OV positive   | 55   | 118  | 173             | 68           |

16 h after infection with L. pneumophila carrying pMMB-GRN, macrophages were incubated for 4 h with IPTG and, where indicated, TR-OV, then colocalization of bacteria, GFP, and LAMP-1 or TR-OV was analyzed by fluorescence microscopy as described in Materials and Methods.

Discussion

A parasite of amebae and macrophages, L. pneumophila escapes a rapid death in phagocytes by preventing phagosome–lysosome fusion. Classic experiments by Horwitz and colleagues determined that during the first 8 h of infection, L. pneumophila phagosomes fail to acidify or acquire lysosomal proteins (2–4). Nascent phagosomes also lack early and late endosomal proteins (5–7, 38, 39). Here, cell biological studies focused on the replication phase of the L. pneumophila intracellular life cycle revealed that the pathogen vacuole changes dramatically with time. Coincident with bacterial replication, a significant proportion of L. pneumophila vacuoles acidify, acquire lysosomal proteases, and accumulate material from the endocytic pathway. Moreover, residence within a lysosomal compartment appears to promote L. pneumophila growth: neutralization of the endosomal compartment inhibited vacuole maturation and bacterial replication.

Several previous observations are consistent with a phagolysosomal residence for replicating L. pneumophila. Bacteria obtained from E broth cultures are degraded in macrophage lysosomes, whereas PE L. pneumophila evade phagosome–lysosome fusion (12). L. pneumophila vacuoles aged >24 h frequently contain granular and membranous material (40–42). DotA, a protein that L. pneumophila requires during infection to arrest phagosome maturation (7, 43), is dispensable during the intracellular replication period (44). Reagents that neutralize the endosomal compartment reportedly prevent L. pneumophila replication in ma-
pherages, HeLa cells, or amebae (32, 37, 45, 46). Finally, C. burnettii, an obligate intracellular pathogen closely related to L. pneumophila (47, 48), replicates within acidic phagolysosomes of macrophages (19–21).

A 1-h treatment with BFA was sufficient to neutralize the macrophage endosomal compartment (Table I), yet it did not block replication of L. pneumophila at the single cell stage (Figs. 4 and 6). Therefore, whereas an acidic pH triggers development of dormant C. burnettii to its metabolically active variant (49), L. pneumophila differentiation from the PE virulent form to the replicative form (12) may be pH independent. However, robust growth of intracellular L. pneumophila did correlate with interaction between the pathogen vacuole and the endosomal compartment (Figs. 4–6).

Byrd and Horwitz postulated that experimental neutralization of the monocyte vacuolar pH inhibits dissociation of iron from endosomal transferrin, starving intracellular L. pneumophila of iron (35, 50). In our experimental model, we were not able to demonstrate that BFA inhibits intracellular L. pneumophila replication by an iron-dependent mechanism. Addition to BFA-treated phagocytes of iron nitritoacetate to 100 μg/ml did not consistently restore L. pneumophila growth (data not shown), although interpretation of this result was complicated as, in the absence of BFA, this compound partially inhibited intracellular bacterial replication and was toxic to macrophages (data not shown). Moreover, L. pneumophila replicated efficiently for 24 h in broth lacking supplemental iron, in macrophages cultured in medium lacking serum, a source of transferrin, or in murine macrophages treated with the iron chelator deferoxamine at concentrations previously shown to inhibit bacterial growth in human monocytes (50; data not shown). Although ferric iron is essential for maximal L. pneumophila growth in broth, the concentration reportedly required varies from trace amounts to ~20 μM (51–55).

Finally, inhibition of proton ATPase activity with specific drugs, such as BFA and the concanamycins, has pleiotropic effects, including deregulation of trans-Golgi trafficking (56, 57), inhibition of autophagy (58), and arrest of endosome maturation (59). Indeed, BFA inhibited not only intracellular L. pneumophila replication, but also fusion of its vacuole with the endocytic network (Fig. 6). Therefore, although it is clear that neutralizing the endosomal pH interferes with L. pneumophila replication, the mechanism of this inhibition warrants more detailed study.

Previously, Horwitz and Maxfield determined that L. pneumophila reside in neutral phagosomes by analyzing vacuoles that were ~6 h old (2); our studies of phagosomes aged 3–6 h (Table I) corroborate their results. They also reported that 18-h phagosomes maintain a neutral pH, a conclusion contradictory to ours. However, their method to measure pH required that the bacteria retain fluorescent antibody opsonins bound before macrophage infection. As bacterial replication is expected to dilute significantly the surface opsonins, their 18-h pH values may represent vacuoles that contained nonreplicating L. pneumophila.

Our observation that macrophage vacuoles harboring replicating L. pneumophila are acidic presents a conundrum, as this pathogen grows optimally in broth of pH 6.3–7.2 (53, 60). However, consistent with a previous report (61), bacteria replicating intracellularly are acid resistant (Figs. 1, 2, 5, and 8). Presumably, during its several hours of isolation from the endocytic pathway, L. pneumophila responds to intracellular cues by inducing expression of factors that promote survival and perhaps replication within this hostile but nutrient-rich compartment. By analogy to other gram-negative bacilli, an early, moderate decline in its vacuolar pH may induce L. pneumophila acid resistance (62), or a variety of noxious conditions encountered during phagocytosis may induce cross-tolerance of L. pneumophila to other environmental stresses (31). In fact, L. pneumophila express heat shock proteins during growth in macrophages (10, 61, 63). Alternatively, acid resistance of L. pneumophila may be promoted by factors present in macrophages, but not ATY, such as high concentrations of amino acids that can be metabolized to produce basic compounds (64). Acid-resistant bacteria may exploit the lysosomal compartment for its ample supply of amino acids, the primary source of carbon and energy for L. pneumophila (65, 66), and membrane, to accommodate numerous progeny.

Aspects of the L. pneumophila intracellular pathway resemble cellular autophagy. During this ubiquitous eukaryotic process, the endoplasmic reticulum engulfs cytoplasmic material and organelles which are then degraded as the autophagosome acquires LAMP-1, the proton ATPase, and lysosomal hydrolases (67). Likewise, by 4–6 h after formation, endoplasmic reticulum surrounds the L. pneumophila vacuole (8); by 12 h, the vacuole begins to acquire lysosomal characteristics. Furthermore, experimental conditions that increase macrophage autophagy also promote association of the endoplasmic reticulum with the pathogen vacuole and modestly stimulate bacterial growth (8). Similarly, vacuoles harboring either replicative Leishmania amastigotes or Brucella abortus acquire features of both the endoplasmic reticulum and the late endosomal compartments (68–72). Perhaps some vacuolar pathogens induce autophagy to obtain nutrients. Alternatively, autophagy may serve as a macrophage quality control mechanism that recognizes aborted phagosomes and delivers these organelles to the lysosomes.

An analogous correlation between microbial growth phase, autophagy, and vacuole maturation has been described for Leishmania. When infectious Leishmania promastigotes are ingested by macrophages, phagosome maturation is blocked by lipophosphoglycan on the parasites’ surface (14). As promastigotes develop into the replicative amastigote form, lipophosphoglycan expression is down-regulated, the vacuole associates with the endoplasmic reticulum and merges with the endosomal pathway, and the parasite replicates within an acidic phagolysosomal vacuole (68, 73, 74).

Because L. pneumophila is amenable to molecular genetics, it may serve as a tool for identifying the mechanisms by which pathogens, including Coxiella and Leishmania, tolerate or exploit the lysosomal environment. Understanding
how \textit{L. pneumophila} manipulates macrophage cellular processes to isolate its nascent phagosome from the endosomal pathway, form an autophagosomal vacuole, then survive and perhaps replicate, within an acidic, lysosomal compartment may elucidate the mechanisms that govern macrophage membrane traffic, as well as the virulence strategies of other intracellular pathogens.

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