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Identification of *Legionella pneumophila* Mutants That Have Aberrant Intracellular Fates

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After uptake by macrophages, *Legionella pneumophila* evades phagosome-lysosome fusion and replicates in a compartment associated with the endoplasmic reticulum. A collection of bacterial mutants defective for growth in macrophages were isolated, and the intracellular fate of each mutant strain was analyzed by fluorescence microscopy. To measure intracellular replication, bacteria inside macrophages were stained with the DNA dye 4′,6-diamidino-2-phenylindole (DAPI). Evasion of the endocytic pathway was quantified by immunofluorescence localization of lgp120 (LAMP-1), a membrane protein of late endosomes and lysosomes, or by measuring colocalization of bacteria with a fluorescent tracer, Texas red-ovalbumin, preloaded into lysosomes. Replication vacuoles were quantified by immunofluorescence localization of BiP, an endoplasmic reticulum protein. By these approaches, four phenotypic groups of mutants were classified. One class formed replication vacuoles less efficiently than the wild type did; another formed replication vacuoles, but replication was abortive; in another class, most phagosomes containing bacteria acquired markers of the endocytic pathway but a minority formed replication vacuoles and the bacteria replicated; finally, a fourth class, the one most defective for intracellular growth, occupied vacuoles that acquired markers of the endocytic pathway.

Legionella pneumophila infects the lungs when aerosols of contaminated water are inhaled by individuals with impaired immune defenses. *L. pneumophila* colonizes alveolar macrophages (16), and its ability to cause disease correlates with its ability to replicate in macrophages (7). *L. pneumophila* grows in freshwater amoebae, apparently a natural reservoir of bacteria, similarly to the way it grows in macrophages.

To establish a productive intracellular infection, *L. pneumophila* must evade the macrophage bactericidal activities and acquire the metabolites needed for replication. Detailed ultrastructural studies of the *L. pneumophila* intracellular pathway by Horwitz and coworkers form the basis for the current understanding of how this gram-negative bacterium establishes a protected niche for growth in the macrophage. *L. pneumophila* is taken up within coils of plasma membrane, which resolve to form a vacuole with a single membrane (17, 19). The phagosome neither acidifies nor fuses with the lysosomes (18, 21). Instead, the cytoplasmic face of the vacuole appears decorated first with small vesicles, next with mitochondria, and then with ribosomes (17). In both macrophages and amoebae, *L. pneumophila* replicates in a compartment bounded by the endoplasmic reticulum (ER) (12, 17, 33). After approximately 24 h, the host cell lyses, releasing the bacteria for a new round of infection (17).

The importance of the association of the *L. pneumophila* phagosome with particular host organelles is supported by morphological analyses of wild-type *L. pneumophila* and mutants defective for intracellular growth. First, association of the ER with the bacterial phagosome has been correlated with intracellular replication in kinetic studies of *L. pneumophila* growth in macrophages (17, 20, 33). Second, *icm* and *dotA* mutants, which do not replicate intracellularly, do not associate with host mitochondria or ER but instead interact with the endocytic pathway (3, 4, 20, 24).

An additional line of evidence in support of a functional role for the interactions between *L. pneumophila* phagosomes and host organelles comes from ultrastructural studies of other intracellular pathogens. For example, *Leishmania donovani* is taken up within coils of macrophage plasma membrane (6), the *Toxoplasma gondii* vacuole associates with mitochondria and ER (22), and phagosomes containing *Mycobacterium tuberculosis* (1, 9) or *Chlamydia psittaci* (15) do not fuse with host lysosomes. These pathogens may utilize similar strategies to establish intracellular infections.

The intracellular route followed by pathogens appears to be critical for virulence. Identification of the microbial factors required at particular steps of the intracellular pathway will facilitate an understanding of how pathogens manipulate host cellular processes to establish a replication niche. Therefore, we have undertaken a genetic analysis of *L. pneumophila* intracellular growth by using macrophages derived from bone marrow cells of A/J mice as a tissue culture model of infection (33, 36). In this paper, we describe the isolation of a collection of mutants defective for intracellular growth and a classification strategy based on the intracellular fate of the mutants in macrophages.

MATERIALS AND METHODS

Cell culture. Mouse macrophages were derived from the bone marrow exudate of female A/J mouse femurs (Jackson Laboratory, Bar Harbor, Maine) as described previously (33). For bacterial infections, macrophages were cultured in RPMI (GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) (RPMI-FBS) without antibiotics in Falcon 24-well or 6-well tissue culture dishes (Becton Dickinson, Lincoln Park, N.J.) or on coverslips (12 mm in diameter, no. 1 thickness; Fisher Scientific) as indicated.

***Legionella* strains and media.** The *L. pneumophila* strains used in this study are derived from the Philadelphia-1 strain, serogroup 1. The strain names have three components: Lp (*Legionella pneumophila*), 0 or 1 (isolated in our laboratory's first or second mutant isolation procedure, respectively), and isolate number. Lp02 (*rpsL*, HsdR$^-$, Thy$^-$) and Lp03 (*dot03 rpsL* HsdR$^-$, Thy$^-$) have been described previously (3); strains Lp112, Lp114, Lp120, Lp126, Lp147, Lp161, and Lp172 were derived from Lp02 as described below. *L. micdadei* D-2676, provided by Barry S. Fields, National Center for Infectious Diseases, is a clinical isolate

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that has been passaged on bacteriological medium less than 10 times. This strain replicates in human monocytes and monocytoid U937 cells, but the yield of CFU is 10- to 50-fold lower than that of *L. pneumophila* Lp02 in these cell models (34). When incubated with A/J mouse bone marrow-derived macrophages, the number of *L. micdadei* CFU decreases approximately 10-fold every 24 h and the intracellular bacteria colocalize with the lysosomal Texas Red-ovalbumin (TRov) probe (34). Thus, *L. micdadei* is avirulent in A/J mouse macrophages.

L. pneumophila strains were maintained as frozen glycerol stocks and were cultured on *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal-yeast extract agar (CYET) or liquid ACES-buffered yeast extract broth (AYET), pH 6.9 (11), supplemented with 100 μg of thymidine per ml of medium as described previously (33). To test for tryptophan and nucleoside auxotrophs, strains were dilution streaked on CAA medium, prepared as described previously (27) with and without 100 μg of L-tryptophan per ml of medium, and supplemented with 100 μg of thymidine per ml of medium.

Bacterial conjugation. Plasmid pMS1 was transferred from *Escherichia coli* SM10(λpir) (31) [F^-::RP4-2-Tc::Mu(λpir)] to *L. pneumophila* by conjugation as described previously (3). Plasmids pMS4 and pMS10 were transferred from *E. coli* DH5α [*supE44* Δ*lacU169* (Φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] to *L. pneumophila* by triparental conjugation with *E. coli* MT607 (*recA56 pro-82 thi-1 hsdR17 supE44*) carrying helper plasmid pRK600 (13). Transconjugants were selected and cultured on plates containing 15 μg of kanamycin per ml of CYET agar. Transfer of extrachromosomal plasmids was verified by isolating plasmid DNA from the recipients and analyzing it by digestion with restriction enzymes.

Genetic complementation of strains Lp147, Lp161, Lp114, and Lp126 by *dotA* was tested. Before strains were constructed for genetic complementation tests, kanamycin-sensitive derivatives of the mutant strains were isolated. To obtain exconjugants of the *dotA* merodiploid strains, which can arise by homologous recombination between pMS1 and genomic *dotA* sequences, the mutants were cultured in AYET without kanamycin selection for approximately 10 to 15 generations. Next, single colonies, obtained by spreading dilutions of each culture on CYET, were replica plated onto CYET and CYET containing 15 μg of kanamycin per ml of agar. Kanamycin-sensitive strains were identified and colony purified on CYET.

DNA manipulations. Plasmid DNA was isolated from *E. coli* and *L. pneumophila* by a standard nonalkaline lysis procedure, followed by sequential precipitations with hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, Mo.) and ethanol as described previously (10). Reactions with calf intestinal alkaline phosphatase, DNA ligase, and restriction enzymes were performed as specified by the suppliers (New England Biolabs, Beverly, Mass.; Boehringer Mannheim, Indianapolis, Ind.).

Plasmids. Plasmid pMS1, which was used to construct *dotA* merodiploids, was derived from pJM703.1 (26), which carries the R6K origin of replication, RP4 mobilization sequences, and a gene encoding resistance to ampicillin. Plasmid pMS1 contains the 3.3-kb *Eco*RI *dotA* fragment isolated from plasmid pKB9 (4) at the unique *Eco*RI site and a 1.1-kb fragment encoding resistance to kanamycin located within the unique *Nru*I site of pJM703.1. Because replication of pMS1 is dependent on expression of the protein pi, it replicates extrachromosomally in the host *E. coli* SM10(λpir) but is maintained in *L. pneumophila* only after integration by homologous recombination into the chromosome.

Plasmids pMS4 and pMS10 were used for *dotA* complementation tests. Plasmid pMS4 is a derivative of plasmid pMMB67EH (14), which carries the RSF1010 origin of replication, RP4 mobilization sequences, and a gene encoding resistance to ampicillin. To construct pMS4, the *lacI*^{q} sequences and the *tac* promoter were deleted from the *Apa*I site of *lacI*^{q} to the *Sal*I site of the multicloning sequence, and a 1.2-kb *Pst*I fragment encoding resistance to kanamycin was inserted at the *Pst*I site of the multicloning sequence. Plasmid pMS10 was constructed by cloning the 5.6-kb *Sph*I *dotA* fragment from plasmid pDOT1 (3) into the unique *Sph*I site of pMS4.

Mutagenesis. To increase the frequency of mutations that affect intracellular growth, *L. pneumophila* Lp02, which served as the wild-type parent for these studies, was treated with ethyl methanesulfonate (Eastman Kodak, Rochester, N.Y.). Strain Lp02 was grown overnight at 37°C to a density of 4×10^8 cells per ml of AYET. A 25-ml culture was collected by centrifugation, washed twice with 37°C phosphate-buffered saline (PBS) supplemented with 100 μg of thymidine per ml, and resuspended in 12.5 ml of 37°C PBS containing thymidine and 1.2% (vol/vol) ethyl methanesulfonate. The bacteria were incubated with aeration for 30 min at 37°C, and the mutagenesis was stopped by addition of 25 ml AYET. The mutagenized cells were collected by centrifugation and resuspended in 25 ml of AYET, and 1-ml aliquots were diluted into culture tubes containing 9 ml of AYET. Next, the 10-ml cultures were incubated with aeration overnight at 37°C to allow expression of mutant phenotypes. By this procedure, 24 pools of mutagenized cells were established.

After a typical mutagenesis, approximately 5% of the initial titer of viable counts was recovered; loss was due to toxicity of the mutagen and inefficient cell collection during the washing procedure. The degree of mutagenesis was monitored by determining the number of CFU on CYET and on CYET containing 5 μg of rifampin per ml. Typically, rifampin-resistant colonies arose at a frequency of 2×10^{-6}, which represents a 100-fold increase in the frequency of resistance relative to an unmutagenized control strain.

Thymineless death enrichment. The pools of mutagenized strains were enriched for intracellular growth mutants by using a thymineless death protocol, as described previously (3). This strategy is based on the observation that *L. pneumophila* thymine auxotrophs that are otherwise competent for intracellular growth do not survive in macrophages cultured in medium lacking thymidine or thymine (27). In contrast, strains that are both thymine auxotrophs and defective for intracellular replication survive in macrophages cultured without supplemental thymine. Pilot experiments performed with wild-type strain Lp02 and *dotA* mutant strain Lp03 indicated that a 10-fold enrichment for the mutant cells could be obtained after a 15- to 20-h incubation with mouse macrophages in medium lacking thymidine (data not shown).

To enrich for mutants, approximately 2×10^7 to 8×10^7 bacteria from each pool were added to 2×10^6 macrophages plated in each well of a Falcon six-well tissue culture plate (Becton Dickinson). After a 17- to 19-h incubation at 37°C in medium without thymidine, extracellular bacteria, which included cells not internalized by macrophages as well as progeny of replication-competent bacteria, were killed by addition of 50 μg of gentamicin per ml of medium. After 30 min, the monolayers were washed three times with medium. The surviving intracellular bacteria were recovered by lysing each macrophage monolayer by trituration with distilled water and culturing the lysates for 3 days on CYET agar. The enrichment was then repeated. Typically, 10^3 to 10^4 bacteria were recovered from each well of macrophages.

Previously, a similar enrichment strategy led to the identification of the *dotA* gene (3). To reduce the likelihood of isolating additional *dotA* alleles, a copy of the wild-type *dotA* gene was transferred by conjugation to the pools of candidate mutant strains. In the pilot mutant isolation procedure that identified mutants Lp161, Lp114, and Lp120, the conjugation was performed prior to the thymineless death enrichment step. In the second mutant isolation procedure, the conjugation step followed the enrichment step.

Phenotypic screens for intracellular growth mutants. Approximately 150 candidate strains from each of 10 pools were tested for the ability to replicate in macrophages by using a plaque assay quick screen, as previously described (3). Macrophages plated at a density of 3×10^6 cells per well of Falcon six-well tissue culture plates and overlaid with RPMI containing 20% FBS, 0.9% molten Noble Agar (Difco), and 100 μg of thymidine per ml were inoculated, using toothpicks, with as many as 75 strains per well. A strain was judged to be a candidate intracellular growth mutant if the zone of macrophage lysis at the point of inoculation was smaller than the plaque made by the parent strain, Lp02. Candidates were colony purified and then tested by the more sensitive single-plaque assay, performed as described previously (3). A range of multiplicities of infection was obtained by infecting cultures of 3×10^5 macrophages per well of a Falcon 24-well tissue culture plate with serial dilutions of bacteria.

Quantification of intracellular bacterial growth. The growth of *L. pneumophila* in macrophage monolayers was measured as described previously (33). Briefly, approximately 5×10^4 CFU was added per well of 2.5×10^5 macrophages. After incubation at 37°C for approximately 2 h, the monolayers were washed and fresh medium was added. After an additional incubation for the periods indicated, the monolayers were lysed by trituration with distilled water. The cellular lysates and culture supernatants were pooled and vortexed, and appropriate dilutions were plated on CYET medium. This procedure was judged to be sufficient to disrupt replication vacuoles, since repeated passage of lysates through a 27-gauge needle released a similar number of CFU (29). The number of CFU per well was determined for duplicate or triplicate wells.

Quantification of bacterial initiation of macrophage infection. Initiation of intracellular infection was assessed by determining the fraction of bacteria that were viable and cell associated after incubation with macrophages for 0.5 h. Contact between the bacteria and the macrophage monolayers was promoted by centrifugation of the tissue culture plates for 10 min at $100 \times g$. After incubation at 37°C for 0.5 h, the monolayers were washed three times with medium and the mean number of CFU associated with the macrophage monolayer was determined from triplicate wells as described for the intracellular growth assay. The number of CFU added to each macrophage monolayer was determined by measuring the titers of duplicate samples of the infection medium on CYET plates. The efficiency of infection initiation was calculated by dividing the number of CFU associated with the monolayer at 0.5 h by the number of CFU added to the monolayer. Under these conditions, 2 to 10% of the wild-type CFU added to a macrophage monolayer was cell associated after 0.5 h (data not shown).

Quantification of intracellular growth by individual bacteria. The ability of individual bacterial cells to replicate intracellularly was assessed by microscopic examination of infected macrophages. Multiplicities of infection of less than 0.1 were ensured by adding serial dilutions of each mutant strain to wells containing macrophages cultured on coverslips. To improve the synchrony of the infection, the mixed cultures were incubated at 37°C for 1 h and then most extracellular bacteria were removed by washing the monolayers three times with medium at 37°C. The cultures were incubated for an additional 14 to 19 h. Under these conditions, most wild-type bacteria had replicated to large numbers and most of the infected macrophages were intact. The infected monolayers were fixed for 20 min with periodate-lysine-paraformaldehyde (PLP) fixative (25) containing 5% sucrose (PLP-sucrose), washed three times with PBS, and then extracted by immersion in −20°C methanol for 10 s. The cells were then washed three times with PBS, and bacterial and macrophage DNA was stained fluorescently by incubating the preparations with 0.1 μg of 4′,6-diamidino-2-phenylindole

(DAPI) per ml of PBS for 5 min at room temperature. Intact bacteria are stained brightly by this technique, while degraded bacteria are not labeled. At least 50 infected macrophages were located, and the number of intact bacteria per macrophage was counted.

Immunofluorescence microscopy. The distribution of markers for the late endosomal and lysosomal compartments and for the ER in macrophages infected with wild-type and mutant *L. pneumophila* was analyzed by immunofluorescence microscopy. For these studies, macrophages were cultured, infected, fixed, stained, and photographed as described previously (33). The interaction of intact *L. pneumophila* with the late endosomal and lysosomal compartment was assessed 1.5 to 2.5 h after infection by immunofluorescence localization of the protein lgp120 (LAMP-1) with a 1:100 dilution of the rat monoclonal antibody IB4. This reagent was developed by Thomas August, Johns Hopkins University School of Medicine, and was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Md., and the Department of Biology, University of Iowa, Iowa City, Iowa, under contract N01-HD-2-3144 from the National Institute of Child Health and Human Development. Colocalization of lgp120 with bacterial phagosomes was interpreted as evidence of fusion between late endosomal or lysosomal vacuoles and the phagosomes. Because only a limited number of markers were examined, these studies did not establish whether *L. pneumophila* phagosomes interacted transiently or merged with the endocytic pathway.

The association of intact *L. pneumophila* with the ER was assayed 4 to 5.5 h after infection by immunofluorescence localization of the ER luminal protein BiP with a 1:250 dilution of a rat monoclonal antibody (23) (a kind gift of David Bole). The anti-lgp120 and anti-BiP antibodies were visualized with a fluorescein-conjugated goat anti-rat immunoglobulin G antibody (Zymed, South San Francisco, Calif.) diluted 1:250. Intracellular and extracellular bacteria were stained differentially as described previously (33). Briefly, prior to permeabilization, the fixed cells were incubated with rabbit serum specific for *L. pneumophila*, which was subsequently stained with rhodamine-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim). After permeabilization, intact intracellular and extracellular bacteria were stained with DAPI as described for the intracellular growth assay.

Phagosome-lysosome fusion assay. The interaction between *L. pneumophila* and the lysosomal compartment was assessed with the soluble fluorescent protein TRov (32). The macrophage endocytic pathway was first labeled by pinocytosis of 50 μg of TRov per ml of RPMI-FBS for 30 min at 37°C. To allow the TRov to accumulate specifically in the lysosomes, the labeling medium was removed, the monolayers were washed with three changes of 37°C medium, and the cells were incubated in RPMI-FBS for an additional 30 min at 37°C. To improve synchronization of the infection and to ensure a multiplicity of infection of less than 0.1, serial dilutions of bacteria were added to the macrophage monolayers and the cultures were centrifuged for 10 min at 100 × *g*. After a 30-min incubation at 37°C, most extracellular bacteria were removed by washing the monolayers three times with RPMI at 37°C. Fresh 37°C medium was added, and the incubation was continued for 1 to 1.5 h or 4.5 to 5.5 h. The infected monolayers were then fixed and stained to identify intracellular bacteria.

In our initial morphological studies, the cultures were fixed for 20 min at room temperature with GF fixative (28) [3.7% formaldehyde, 0.05% glutaraldehyde, 250 mM sucrose, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), 0.5 mM EDTA, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)] prewarmed to 37°C. Then, extracellular and intracellular bacteria were stained differentially, as described above, with a fluorescein-conjugated goat anti-rabbit immunoglobulin G and DAPI. This protocol preserved the fluorescent labeling of the lysosomal compartment of methanol-extracted cells (35).

Quantitative studies of phagosome-lysosome fusion were performed in essentially the same manner, with two modifications. First, preferential staining of phagolysosomes was obtained by fixing the cells with PLP-sucrose. This fixative preserved the lysosomal compartmentalization of the TRov probe, provided that the cells were not methanol extracted (data not shown). Permeabilization of the preparations, which was required to stain intracellular bacteria, revealed that TRov was more efficiently cross-linked to lysosomal vacuoles containing bacteria than to those without bacteria. In particular, the TRov was removed from the permeabilized lysosomal compartments but not from lysosomal bacteria, when the cells were washed three times with PBS after methanol extraction.

A second modification of the protocol was necessary because partially degraded bacteria did not stain brightly with DAPI (data not shown). Extracellular bacteria were stained with rabbit serum specific for *L. pneumophila* and Cascade Blue-conjugated goat anti-rabbit immunoglobulin G antibody (Molecular Probes, Eugene, Oreg.), diluted 1:5,000. The preparations were then extracted with methanol, and both intracellular and extracellular bacteria were stained with *L. pneumophila*-specific antiserum and fluorescein-conjugated anti-rabbit IgG. By this method, intracellular bacteria were green and extracellular bacteria were both blue and green.

The efficiency of phagosome-lysosome fusion was calculated by dividing the number of intracellular bacteria that colocalized with TRov by the total number of intracellular bacteria. Intracellular bacteria were defined as rod- and non-rod-shaped particles that were stained by *L. pneumophila*-specific antiserum. Lysosomal bacteria were often fragmented, especially after a 5- to 6-h incubation. Therefore, a multiplicity of infection of less than 0.1 was used for these experiments, and multiple antigenic fragments contained within a single macrophage were scored as one bacterium. Formalin-fixed Lp02 cells prepared as described previously (17) and viable but avirulent *Legionella micdadei* cells (see above) served as the negative control strains.

Efficient evasion of the macrophage lysosomes by wild-type *L. pneumophila* was observed when the bacteria were grown to a thick-lawn stage on CYET plates (24 to 36 h) or when grown past log phase in AYET broth prior to infection. In contrast, wild-type cells which had formed thin patches on CYET plates (grown less than 24 h) or those in early log phase in AYET broth frequently colocalized with lysosomal TRov, and after fixation and methanol extraction, many extracellular bacteria were rounded rather than rod shaped. Culture conditions apparently affected the physical characteristics and, in turn, the intracellular fate of *L. pneumophila*. Therefore, all of the strains were grown to the thick patch stage on CYET plates prior to analysis; under these conditions, the majority of the extracellular bacteria of each of the strains were rod shaped after fixation and methanol extraction (data not shown).

Other phenotypic tests. To test for sensitivity to NaCl, strains were grown overnight on CYET plates and then suspended in sterile water, and the titers of serial dilutions were measured for determination of viable counts on CYET plates and CYET plates containing 0.6% NaCl. To test for sensitivity to tissue culture medium, bacteria grown overnight on CYET plates were suspended in AYET broth and then diluted into AYET containing no serum, 10% FBS, or 25% FBS. The cultures were incubated with aeration at 37°C for 1 h, and the titers of serial dilutions were measured for determination of viable counts on CYET medium.

The integrity of the membranes of the mutant and wild-type strains was compared by two methods. Wild-type *L. pneumophila* cells are sensitive to the nonionic detergent Triton X-100: after incubation with 0.01% detergent in PBS, the number of CFU decreases 100-fold. To determine whether the mutants were more sensitive to this detergent, bacteria grown overnight on CYET were suspended in PBS, diluted into 0.01, 0.005, or 0.001% Triton X-100 in PBS, and incubated for 5 min at room temperature, and then the titers of serial dilutions were measured for determination of viable counts on CYET medium. To assess whether the mutants were sensitive to high osmolarity, bacteria grown overnight on CYET plates were suspended in AYET broth and then diluted into AYET containing no additional salt, 150 mM KCl, or 300 mM KCl. The cultures were incubated with aeration at 37°C for 1 h, and the titers of serial dilutions were measured for determination of viable counts on CYET medium.

RESULTS

Isolation of intracellular growth mutants. The aim of this study was to isolate mutants of *L. pneumophila* defective for formation of the replication vacuole. Such mutants should also be defective for intracellular growth (3, 20, 33). Therefore, we first isolated mutants that do not replicate as well as the wild type in macrophages, and we then studied the intracellular fate of each mutant.

To increase the frequency of mutations that alter *L. pneumophila* intracellular traffic, the wild-type strain Lp02 was treated with ethyl methanesulfonate. To enrich the mutagenized populations for mutants defective for intracellular growth, pools of bacteria were subjected to an intracellular thymineless death protocol, which was used previously to isolate the *dotA* locus (3) (see Materials and Methods). To reduce the probability of isolating new *dotA* alleles, plasmid DNA encoding the wild-type *dotA* gene was integrated into the chromosome of the candidate mutants prior to phenotype screening.

Approximately 150 colonies per pool were tested for growth in macrophages by a plaque assay quick screen. Strains that appeared partially or fully defective for intracellular growth were colony purified and then retested for growth in macrophages by a more sensitive single plaque assay. As judged by this technique, approximately 1% of the strains screened exhibited intracellular growth defects. In particular, seven *L. pneumophila* strains defective for growth in macrophages were isolated from seven separate pools: strains Lp112 and Lp172 formed plaques that were smaller than wild-type plaques, while strains Lp147, Lp120, Lp161, Lp114, and Lp126 did not form macroscopic plaques (data not shown). The intracellular growth and trafficking phenotypes of these strains were studied further.

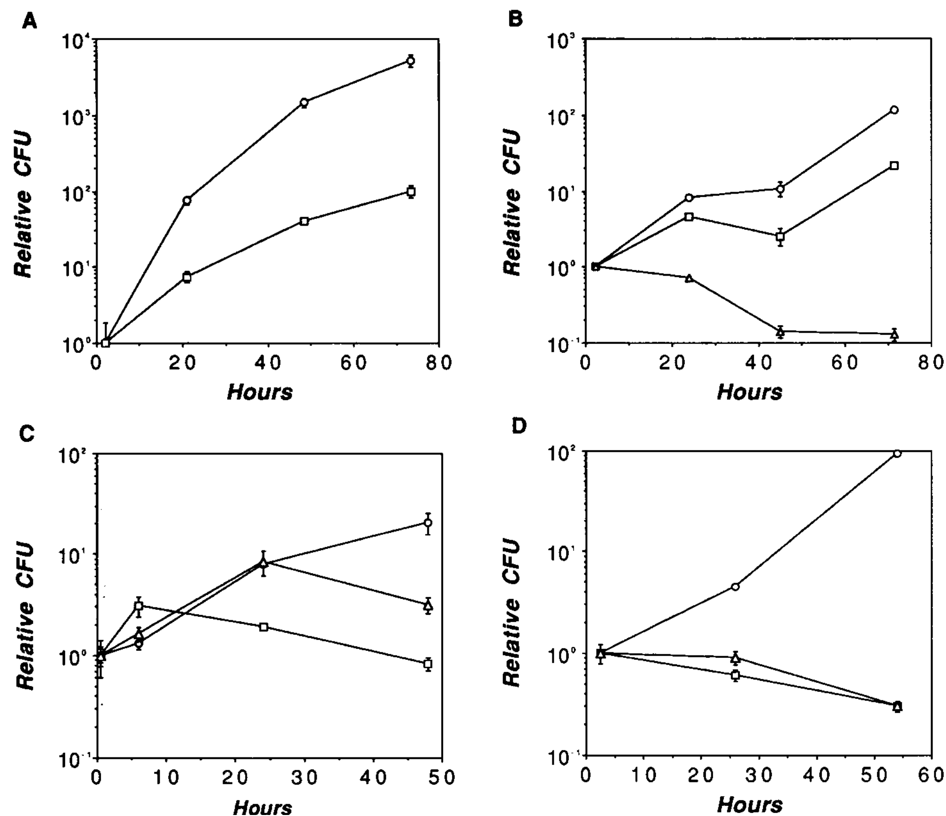

FIG. 1. Intracellular growth phenotype of *L. pneumophila* mutant strains. Mouse macrophages were infected with wild-type or mutant bacteria for the times indicated, and the number of viable bacteria was determined for each strain over the next 2 or 3 days, as described in Materials and Methods. (A) Wild-type strain Lp02 (open circles) and mutant Lp112 (open squares); (B) wild-type strain Lp02 (open circles) and mutants Lp172 (open squares) and Lp126 (open triangles); (C) wild-type strain Lp02 (open circles) and mutants Lp147 (open squares) and Lp120 (open triangles); (D) wild-type strain Lp02 (open circles) and mutants Lp161 (*dotA161*) (open squares) and Lp114 (*dotA114*) (open triangles). The relative number of CFU was calculated by dividing the mean number of CFU at each time point by the mean number of CFU at the first time point for each strain. The mean number of CFU at the first time point for each of the strains was between 1×10^3 and 5×10^4. The number of CFU was determined from duplicate (A, B, and D) or triplicate (C) wells; the standard deviation of the mean is indicated by an error bar for each point. Similar results were obtained in at least two other experiments.

Intracellular growth phenotype of the mutants. The time course of intracellular growth of each of the mutant strains was determined. The yield of the wild-type strain Lp02 typically increased about 100-fold after a 2- or 3-day incubation (Fig. 1). As expected, the two mutants that formed small plaques on macrophage monolayers had the least pronounced defects: the yields of mutants Lp112 (Fig. 1A) and Lp172 (Fig. 1B) were typically about 10-fold lower than those of the wild type. Three of the mutants were severely defective for intracellular growth. The yield of mutant Lp126 decreased about 10-fold during the incubation period (Fig. 1B), while the yield of Lp161 and Lp114 decreased slightly (Fig. 1D). Two of the mutants had more complex phenotypes. For mutant Lp147, the number of CFU after 2 or 3 days of infection was similar to the initial number of CFU; however, this mutant appeared to initiate intracellular replication (Fig. 1C). For mutant strain Lp120, the yield of viable counts consistently increased early in the incubation but not at later times (Fig. 1C). These results indicate that mutants with a range of intracellular growth defects can be isolated after thymineless death enrichment of a chemically mutagenized population of *L. pneumophila.*

The reduced bacterial yields observed in quantitative growth experiments (Fig. 1) could result from defects in extracellular survival, association with macrophages, or intracellular replication. None of the strains was more sensitive than the wild type to components of the tissue culture medium (see Materials and Methods; also data not shown). In addition, the relative number of bacterial CFU associated with the macrophages early after infection was similar for the mutant and wild-type strains (see Materials and Methods; also data not shown). Therefore, the mutants were most probably defective for intracellular replication.

***dotA* genetic complementation tests.** The *L. pneumophila dotA* locus, identified in a previous genetic study, is required for proper targeting and replication of the bacteria (3). To determine whether *dotA* mutants were among those isolated here, genetic complementation tests were performed. Two of the strains appeared to be *dotA* mutants. The intracellular growth defects of the *dotA* mutant strain Lp03 (3) and mutants Lp161 and Lp114 were partially complemented by a plasmid-encoded *dotA* gene. The yield of the partial diploid strains increased at least 10-fold after 3 days, whereas the yield of each strain carrying the parent vector did not increase at all (Table 1). Consistent with these results, full-length *dotA* protein was not present in protein extracts prepared from the two mutants, as judged by immunoblot analysis performed with *dotA*-specific antiserum (29).

Intracellular replication of individual mutant cells. Decreased intracellular bacterial growth determined by time course experiments may indicate either that all of the cells in a population replicate at a subnormal rate or that a subset of the population replicates at the normal rate. To distinguish be-

TABLE 1. *dotA* genetic complementation test

Strain	Plasmid[a]	Mean no. of CFU[b] after: 2 h	Mean no. of CFU[b] after: 72 h	Ratio[c]	Complemented by *dotA*[d]
Lp02	pMS4	9×10^3	6.1×10^5	68	NA
	pMS10	9×10^3	3.7×10^5	41	
Lp03	pMS4	8×10^3	1.1×10^4	1.4	+
	pMS10	8×10^3	7.8×10^4	10	
Lp126	pMS4	2.2×10^4	3.2×10^4	1.5	−
	pMS10	2.5×10^4	4.0×10^4	1.6	
Lp147	pMS4	2.8×10^4	2.2×10^4	0.8	−
	pMS10	2.4×10^4	4×10^3	0.2	
Lp161	pMS4	2.0×10^4	2.6×10^4	1.3	+
	pMS10	1.7×10^4	2.8×10^5	16	
Lp114	pMS4	1.3×10^4	1.0×10^4	0.8	+
	pMS10	7×10^3	2.9×10^5	41	

[a] Plasmid pMS4 is the parent vector; plasmid pMS10 encodes *dotA*.
[b] Mean number of CFU associated with macrophages 2 h after infection and mean number of CFU per well after a 72-h incubation (see Materials and Methods).
[c] Increase in the yield of bacteria, expressed as the mean number of CFU at 72 h divided by the mean number of cell-associated CFU at 2 h.
[d] NA, not applicable. The *dotA* genotypes of strains Lp02 and Lp03 are known to be wild type and mutant, respectively.

tween these two possibilities, the ability of individual bacteria to replicate intracellularly was analyzed microscopically 15 to 20 h after infection of macrophages at a low multiplicity of infection. For the wild-type strain Lp02, nearly 60% of the infected macrophages contained bacteria too numerous to count (more than 20); only rarely did a macrophage contain a single bacterium (Fig. 2A). Similar results were obtained with mutants Lp112 and Lp172, although fewer cells yielded more than 20 progeny (Fig. 2A). Strain Lp147 exhibited a more severe replication defect: although more than 90% of the bacteria replicated, fewer progeny were produced (Fig. 2B). For strains Lp161 (*dotA161*), Lp114 (*dotA114*), and Lp126, approximately 90% of the macrophages contained only a single bacterium and no cells yielded more than 10 progeny after the incubation period (Fig. 2C). A more complex pattern was seen for mutant Lp120. Half of the macrophages contained a single bacterium; however, nearly one-third of the cells generated progeny too numerous to count. This microscopic analysis indicated that for one of the strains, cells express one of two distinct phenotypes: most Lp120 cells were replication defective but a minority were replication competent. For the other strains, the expression of the mutant phenotype was more uniform.

Evasion of the endocytic pathway. Previous studies demonstrated that wild-type *L. pneumophila* phagosomes do not acquire endocytic markers (3, 9, 18) whereas mutant phagosomes do colocalize with endocytic markers (3, 20). Interaction of the new mutants with the endocytic pathway was quantified by immunofluorescence localization of lgp120 (LAMP-1), a membrane protein of late endosomes and lysosomes.

As expected, most phagosomes containing wild-type bacteria did not colocalize with the late endosomal and lysosomal marker: more than 80% of the wild-type phagosomes were lgp120 negative (Fig. 3C and D; Table 2). Similar results were obtained for the mutants least defective for intracellular growth: more than 70% of the Lp112 and Lp172 cells resided in an lgp120-negative compartment (Fig. 3A and B; Table 2). Mutant Lp147, which initiates but does not sustain intracellular replication (Fig. 1C and 2B), also interacted infrequently with the endocytic pathway: 30% of the phagosomes colocalized with lgp120 (Table 2). In contrast, phagosomes containing mutants with the most severe intracellular growth defects were frequently stained brightly by antibodies specific for lgp120. For these mutants, Lp161 (*dotA161*), Lp114 (*dotA114*), and Lp126, 80% of the phagosomes colocalized with the endocytic marker (Fig. 3E to L; Table 2). Mutant Lp120 occupied an lgp120-positive compartment at a similarly high frequency (Table 2), consistent with the observation that more than half of the Lp120 cells are defective for intracellular replication (Fig. 2B).

To determine whether any of the mutants fused with the lysosomal compartment, the soluble endocytic probe TRov was used to label the macrophage lysosomal network by pinocytosis prior to infection (32, 35) (see Materials and Methods). Because the dense macrophage lysosomal network made quantification of phagosome-lysosome fusion difficult (35), infected cells were fixed and stained by a procedure which preferentially labeled phagolysosomes (see Materials and Methods). By this

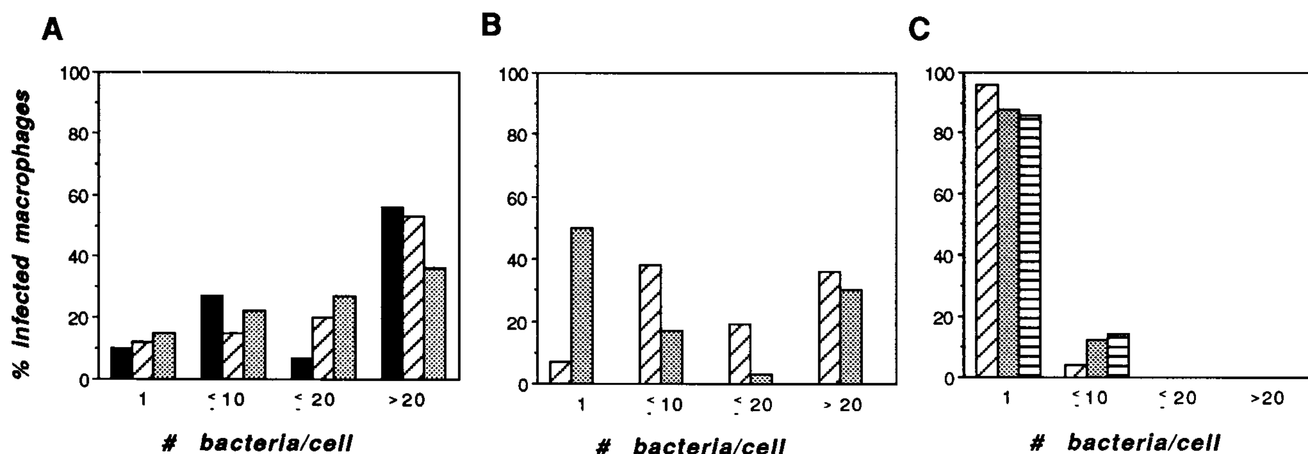

FIG. 2. Intracellular growth phenotype of individual mutant *L. pneumophila* cells. Mouse macrophages were incubated with a low multiplicity of wild-type or mutant bacteria, incubated for 20 h, fixed, and stained with the DNA dye DAPI. Infected macrophages were located, and the number of intact bacteria per macrophage was determined. Bars represent the fraction of 50 or more infected macrophages that contained the indicated number of bacteria. (A) Wild-type strain Lp02 (solid bars) and mutants Lp112 (hatched bars) and Lp172 (dotted bars); (B) mutants Lp147 (hatched bars) and Lp120 (dotted bars); (C) mutants Lp161 (*dotA161*) (hatched bars), Lp114 (*dotA114*) (dotted bars), and Lp126 (horizontally striped bars). Similar results were obtained in one to three other experiments.

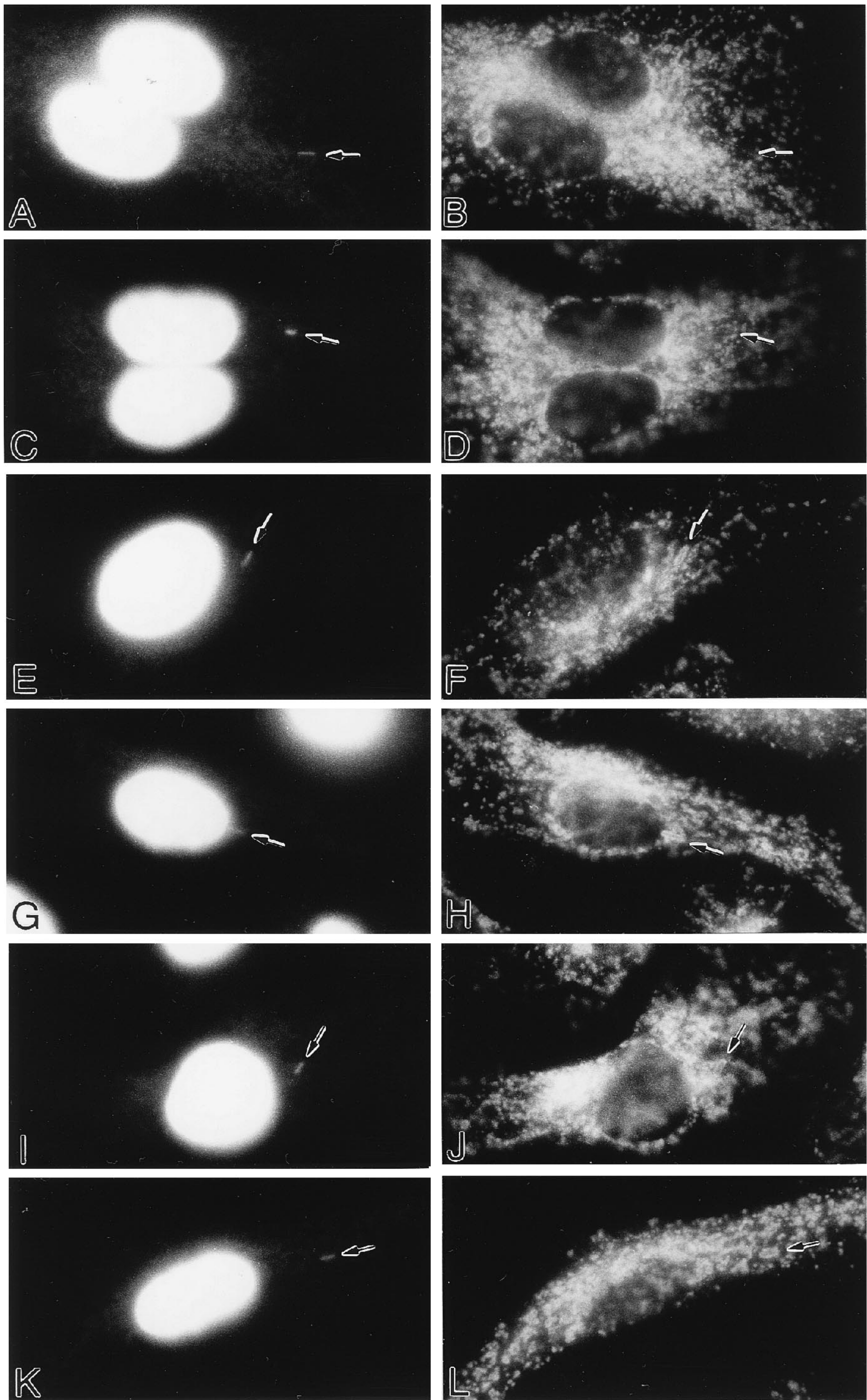
A
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FIG. 3. Immunofluorescence localization of the late endosomal and lysosomal protein lgp120 in macrophages infected with *L. pneumophila*. Mouse macrophages were incubated with wild-type or mutant bacteria for 2 h, fixed, and stained with DAPI to localize DNA of macrophages and intact bacteria (A, C, E, G, I, and K) or with a monoclonal antibody specific to lgp120 to identify the macrophage late endosomes and lysosomes (B, D, F, H, J, and L). Phagosomes formed by wild-type Lp02 (C and D) or the intracellular growth mutant Lp112 (A and B) did not colocalize with lgp120, whereas phagosomes containing Lp161 (*dotA161*) (E, F, K, and L) or mutant Lp114 (*dotA114*) (G, H, I, and J) stained brightly with lgp120-specific fluorescence. Arrows indicate positions of bacteria.

technique, nearly 70% of formalin-killed *L. pneumophila* cells colocalized with the lysosomal marker (Fig. 4I and J; Table 2). In contrast, less than 30% of the wild-type phagosomes colocalized with TRov 5 to 6 h after infection (Fig. 4A, B, E, F; Table 2).

This method was used to analyze the mutants most defective for intracellular growth. Consistent with its frequent residence in an lgp120-negative compartment (Table 2), mutant Lp147 appeared to evade fusion with the lysosomes as efficiently as wild-type *L. pneumophila* did (Table 2). One of the *dotA* mutants, strain Lp161 (*dotA161*), rarely colocalized with TRov (Fig. 4G and H; Table 2). In contrast, two mutants with strong intracellular growth defects frequently colocalized with TRov. At 1 to 2 h after infection, approximately 40% of the phagosomes containing mutants Lp114 (*dotA114*) or Lp126 stained with TRov (Fig. 4K and L, and M and N, respectively); after 5 to 6 h, more than 60% of the phagosomes were stained (Table 2). Mutant Lp120 also fused with TRov-labeled vacuoles, although less frequently. After 1 to 2 h of infection, 30% of the intracellular bacteria colocalized with TRov; after 5 to 6 h of infection, 50% did (Fig. 4O and P; Table 2). As expected for lysosomal degradation of bacteria, strains that colocalized with TRov frequently appeared fragmented (Fig. 4K, L, O, and P), and fragmentation increased over time (data not shown). In general, phagosomes containing the mutants with the most severe intracellular growth defects acquired endocytic markers more frequently than did phagosomes containing replication-competent bacteria.

TABLE 2. Intracellular trafficking of wild-type and mutant *L. pneumophila*

Strain	% lgp120 positive[a]	% TRov positive after[b]:		% BiP positive[c]
		1–2 h	5–6 h	
Lp02	17.7 ± 2.1	17.5 ± 4.0	27.6 ± 4.3	64.8 ± 13.4
Lp112	25.3 ± 5.4	ND[d]	ND	52.0 ± 6.5
Lp172	29.8 ± 4.1	ND	ND	44.3 ± 16.4
Lp147	30.0 ± 5.3	17.0 ± 6.1	19.0 ± 3.1	75.0 ± 7.1
Lp120	79.7 ± 4.5	31.9 ± 4.4	49.5 ± 4.8	15.7 ± 6.1
Lp161	81.0 ± 2.6	9.4 ± 3.8	23.0 ± 3.5	5.0 ± 1.0
Lp114	82.3 ± 2.6	44.9 ± 4.4	66.6 ± 9.2	7.7 ± 3.1
Lp126	86.0 ± 4.4	43.3 ± 7.9	60.2 ± 8.3	4.5 ± 0.5
fk-Lp02[e]	ND	59.7 ± 7.9	68.4 ± 6.7	ND

[a] Macrophages infected for 1.5 to 2.5 h with the bacterial strains indicated were stained for the late endosomal and lysosomal protein lgp120 and intracellular bacteria as shown in Fig. 3 and described in Materials and Methods. Values represent the mean fraction of 20 or more intact intracellular bacteria that were outlined with lgp120-specific fluorescence as determined in three (Lp112, Lp147, Lp120, Lp161 [*dotA161*], Lp114 [*dotA114*], and Lp126), four (Lp172), or seven (Lp02) experiments; the standard error of each mean is shown.

[b] Macrophages labeled by pinocytosis of the endocytic marker TRov were infected for the times indicated with the bacterial strains listed, and phagosome-lysosome fusion was quantified by fluorescence microscopy as shown in Fig. 6 and described in Materials and Methods. Values represent the mean fraction of 50 or more intracellular bacteria that colocalized with TRov determined in three (Lp161 [*dotA161*] and Lp114 [*dotA114*]), four (Lp147 and Lp120), six (Lp02 and Lp126), or seven (formalin-killed Lp02) experiments; the standard error of each mean is shown.

[c] Macrophages infected for 4 to 5.5 h with the bacterial strains indicated were stained for the ER protein BiP and intracellular bacteria as described in Materials and Methods. Values represent the mean fraction of 30 or more intact intracellular bacteria that were outlined with BiP-specific fluorescence in two (Lp161 [*dotA161*] and Lp126), three (Lp112, Lp172, Lp147, Lp120, and Lp114 [*dotA114*]), or six (Lp02) experiments; the standard deviation of each mean is shown.

[d] ND, not determined.

[e] Formalin-killed Lp02 served as the negative control strain in the phagosome-lysosome fusion assay.

Formation of the replication vacuole. Association of the *L. pneumophila* phagosome with the host ER appears to be required for intracellular replication (3, 17, 20, 33). Therefore, we determined whether the mutants formed this specialized vacuole, as judged by immunofluorescence localization of the ER luminal protein BiP (33).

After a 5-h infection of macrophages, 65% of intracellular wild-type bacteria associated with the ER (Table 2). The two strains with the least pronounced growth defects appeared to be partially defective for formation of the replication vacuole. After 5 h, approximately 50% of Lp112 and 45% of Lp172 bacteria associated with the ER (Table 2). Mutant Lp147, which initiates replication at a high frequency (Fig. 2B), formed replication vacuoles as often as the wild type did (Table 2). In contrast, vacuoles containing the mutants with the most severe intracellular growth defects rarely stained with the ER protein BiP: less than 10% of the phagosomes containing the mutants Lp161 (*dotA161*), Lp114 (*dotA114*), or Lp126 were stained with BiP-specific fluorescence (Table 2). For mutant Lp120, about 15% of the intracellular bacteria colocalized with the ER marker after 5 h (Table 2). This result was generally consistent with the observation that after 20 h, approximately 30% of the cells had replicated (Fig. 2B). The results of these experiments are consistent with those of previous reports correlating intracellular replication with the association of the phagosome with the ER (3, 17, 20, 33).

Analysis of the mutants for other phenotypes. The intracellular fate of *L. pneumophila* might be affected by mutations that affect processes important primarily for extracellular growth or survival. To determine whether any of the mutants exhibited extracellular growth defects too subtle to detect with standard media, colony formation was examined under a variety of conditions.

On CYET medium, six of the seven mutants formed colonies that were the same size as or slightly larger than those of the wild-type strain Lp02. Mutant Lp126 consistently formed colonies that were somewhat smaller than wild-type colonies (data not shown). Similar results were obtained when the strains were dilution streaked on CAA minimal plates, which are deficient in tryptophan and nucleosides (see Materials and Methods; also data not shown). Therefore, the aberrant fate in macrophages of at least one of the strains (mutant Lp126) may result from a defect in a pathway that is not dedicated exclusively to intracellular growth.

None of the mutants appeared to be sensitive to components of tissue culture medium, including 10 or 25% FBS (see Materials and Methods; also data not shown). The mutants did not appear to have gross defects in membrane integrity, as judged by their relative sensitivity to the detergent Triton X-100 (see Materials and Methods; also data not shown). Membrane integrity was also assessed by determining the relative sensitivity of the mutants to high osmolarity (see Mate-

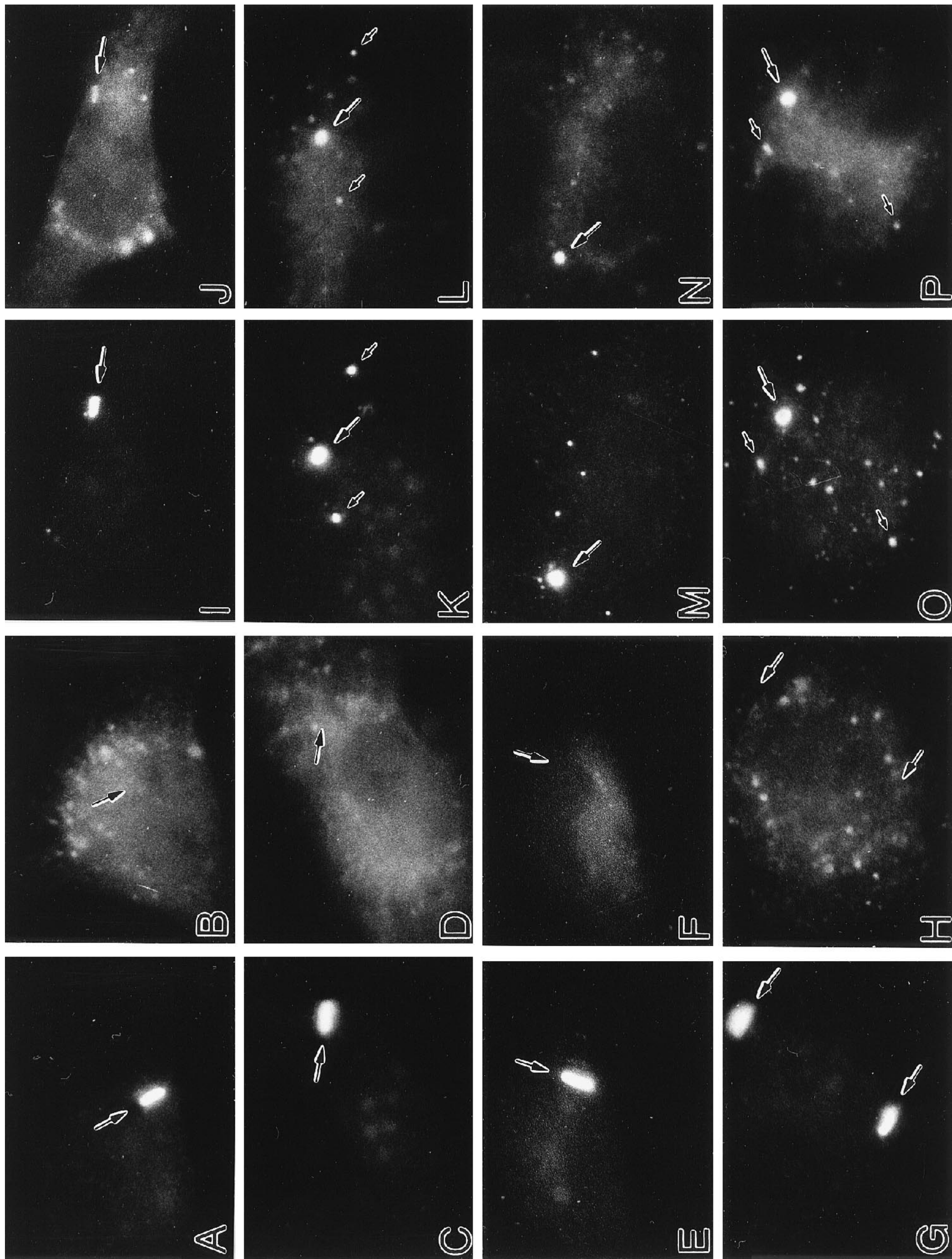

FIG. 4. Phagosome-lysosome fusion visualized in macrophages labeled by pinocytosis of TRov and fixed with PLP-sucrose. Macrophages labeled with the endocytic probe TRov (B, D, F, H, J, L, N, and P) were infected with bacteria for 2 h (A to D, I to N) or 5 h (E to H, O, and P), and the preparations were fixed with PLP-sucrose, methanol extracted, and stained with *L. pneumophila*-specific antiserum to localize intact and degraded intracellular bacteria (A, C, E, G, I, K, M, and O). Phagosomes containing wild-type strain Lp02 (A and B, E and F), mutant Lp147 (C and D), or mutant Lp161 (*dotA161*) (G and H) did not colocalize with lysosomal TRov. In contrast, formalin-killed *L. pneumophila* (I and J), mutant Lp114 (K and L), mutant Lp126 (M and N), and mutant Lp120 (O and P) colocalized with lysosomal TRov. Arrows indicate positions of bacterial particles, and small arrows indicate fragments of bacteria which appeared to result from lysosomal degradation of *L. pneumophila*.

rials and Methods). In these experiments, strains Lp147 and Lp114 exhibited an average fourfold decrease in colony formation relative to the wild-type strain Lp02 after incubation with 300 mM KCl, suggesting that the structural properties of these cells may be somewhat altered.

Analysis of a large collection of *L. pneumophila* mutants defective for killing macrophage-like HL60 cells (*mak* mutants) revealed a strong correlation between the ability to grow intracellularly and sensitivity to sodium chloride (30). In a similar assay, only mutant Lp147 retained the NaCl sensitivity

that is characteristic of virulent *L. pneumophila*. For strains Lp02 and Lp147, the relative plating efficiency in the presence of 0.6% NaCl was less than 0.1%. For the other mutant strains, the plating efficiency in the presence of 0.6% NaCl was 2 to 3 log units higher than that of the wild-type parent strain Lp02 (see Materials and Methods; also data not shown). The functional significance of the *L. pneumophila* response to NaCl is not yet understood.

DISCUSSION

After uptake by macrophages, virulent *L. pneumophila* cells evade the lysosomes and replicate in phagosomes associated with host ER. We have isolated a collection of mutants defective for growth in macrophages and have placed the mutants into four phenotypic groups according to their intracellular fate in macrophages. The phenotypes of these mutants attest to the functional significance of evasion of the endocytic pathway and association with the ER for subsequent growth of *L. pneumophila* in macrophages.

Mutants in the first phenotypic group, Lp112 and Lp172, had the least pronounced defects in intracellular growth and evaded the lysosomes nearly as well as the wild type did but associated with the ER less frequently (Table 2). These strains resembled a previously characterized *L. pneumophila* mutant, Lp046 (3, 33). Mutants in this class may lack a factor(s) important for formation or maintenance of the replication vacuole.

The second phenotypic class is represented by strain Lp147, which evaded the endocytic pathway and associated with the ER nearly as efficiently as wild-type *L. pneumophila* did (Table 2). Approximately 90% of the bacteria initiated intracellular replication (Fig. 2B), but these cells did not establish a productive infection (Fig. 1C). This phenotype resembles that observed for ThyA auxotrophs (3, 27). Mutant Lp147 may be defective for acquisition or synthesis of a particular factor required in the intracellular environment, so that replication is limited by the size of the metabolic stores of each bacterium.

The class of mutants with the most severe intracellular growth defects frequently interacted with the endocytic pathway but not with the ER. Mutant Lp161 (*dotA161*) phagosomes acquired a late endosomal marker (Fig. 3; Table 2), while phagosomes containing Lp114 (*dotA114*) and Lp126 cells acquired both late-endosomal and lysosomal markers (Fig. 3; Table 2). Because a limited number of markers for the endocytic pathway were analyzed, this study has not established whether phagosomes containing mutant *L. pneumophila* merge or interact transiently with endocytic compartments.

Mutants Lp161 and Lp114 were complemented by *dotA*, a gene previously shown to be required for proper intracellular targeting and intracellular growth of *L. pneumophila* (3, 4). While the intracellular growth phenotypes of these *dotA* strains were quite similar (Fig. 1D and 2C), the strains appeared to interact with endocytic compartments to different degrees. Interestingly, similar conclusions were drawn from previous ultrastructural studies of two other *dotA* mutants (3, 4). However, since the Lp161 (*dotA161*) and Lp114 (*dotA114*) mutations were induced chemically, it is possible that a second mutation contributes to one of the mutant phenotypes.

Two *dotA* mutants were isolated in this study (Table 1) in spite of efforts to avoid additional *dotA* mutants (see Materials and Methods). In particular, when *dotA* merodiploids were constructed prior to the thymineless death enrichment step, *dotA* mutants were isolated. This suggests that strains with two defective *dotA* alleles were generated by gene conversion events and selected during the enrichment procedure.

The third member of this phenotypic class, mutant Lp126, died in macrophages, consistent with its frequent colocalization with the lysosomal marker, TRov. Because the thymineless death enrichment favors isolation of mutants which survive in macrophages, we did not expect to identify mutants of this type. However, since the yield of Lp126 decreases only slightly during the first 24 h of infection (Fig. 1B), this strain would survive the enrichment.

The fourth mutant class is represented by strain Lp120, which exhibited the most complex phenotype. Microscopic analysis indicated that the majority of the intracellular bacteria acquired markers from the endocytic pathway whereas a minority of cells associated with the ER (Table 2). Consistent with these colocalization studies, more than half of the intracellular bacteria appeared defective for replication whereas a minority replicated to large numbers (Fig. 2B). The Lp120 mutation might partially reduce the activity of a factor to near the threshold required for virulence. Thus, the majority of the cells contain subthreshold levels and express a mutant phenotype whereas a minority are above the threshold and replicate at normal rates. By this model, mutant Lp120 would be deficient in an activity required for evasion of the endocytic pathway.

The yield of intracellular Lp120 bacteria was comparable to the wild-type yield for the first 24 h, but thereafter it did not increase (Fig. 1C). Perhaps Lp120 expresses an essential factor during growth on bacteriological medium but not in macrophages. Alternatively, Lp120 cells may be more sensitive than wild-type cells to a macrophage cellular response expressed hours after the bacterial infection.

In this study, we quantified the intracellular bacterial replication by two methods (Fig. 1 and 2). Microscopic analysis of wild-type *L. pneumophila* growth in macrophages indicated that a single bacterium frequently generated dozens of progeny in a 20-h infection whereas the number of CFU increased about 10-fold in this same period. In addition, the increased yield of CFU for strain Lp120 was greater than expected from the results of the microscopic assay. The CFU assay quantified growth in monolayers of 10^5 macrophages, and the microscopic assay reported growth in less than 100 macrophages; however, the results of both assays were reproducible. We do not yet understand the basis for this discrepancy. Perhaps a greater portion of Lp120 progeny than wild-type progeny which are visualized microscopically are competent to form colonies under the experimental conditions used here.

The utility of characterizing the intracellular fate of bacterial mutants is illustrated by comparing the phenotypes of strains Lp161 (*dotA161*) and Lp147. In quantitative growth experiments, both strains were severely defective for intracellular growth (Fig. 1C and D). However, microscopic analysis revealed quite different intracellular fates. Mutant Lp161 (*dotA161*) phagosomes acquired a late endosomal marker (Fig. 3; Table 2), and the cells rarely divided (Fig. 2C). In contrast, mutant Lp147 phagosomes associated with the ER (Table 2), and most of the cells replicated (Fig. 2B). These mutants belong to different complementation groups (Table 1) (34). On the basis of their distinct intracellular fates, the corresponding gene products are likely to act in different bacterial pathways.

A number of *L. pneumophila* loci that appear to be important for the virulence of the organism have been described (2, 3, 5, 8, 20, 24, 30). While *icm* and *dotA* mutants have been shown to interact with the endocytic pathway and not with the ER (3, 4, 20, 24), the effect of mutations in the other loci on *L. pneumophila* intracellular fate has not been described. Whether the mutations in the strains characterized in this

study are alleles of previously identified loci other than *dotA* remains to be determined.

This work has demonstrated the value of quantitative fluorescence microscopic assays to assess the intracellular fate of wild-type and mutant *L. pneumophila* in macrophages. Knowledge of the pathway followed by each of the avirulent mutants facilitated classification of the mutants into phenotypic groups. Now, representative strains from each phenotypic group can be chosen for further genetic and molecular analyses of *L. pneumophila* growth in macrophages.

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