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Intracellular Growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a System for Genetic Analysis of Host-Pathogen Interactions

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Conditions were established in which *Legionella pneumophila*, an intracellular bacterial pathogen, could replicate within the unicellular organism *Dictyostelium discoideum*. By several criteria, *L. pneumophila* grew by the same mechanism within *D. discoideum* as it does in amoebae and macrophages. Bacteria grew within membrane-bound vesicles associated with rough endoplasmic reticulum, and *L. pneumophila dot*/*icm* mutants, blocked for growth in macrophages and amoebae, also did not grow in *D. discoideum*. Internalized *L. pneumophila* avoided degradation by *D. discoideum* and showed evidence of reduced fusion with endocytic compartments. The ability of *L. pneumophila* to grow within *D. discoideum* depended on the growth state of the cells. *D. discoideum* grown as adherent monolayers was susceptible to *L. pneumophila* infection and to contact-dependent cytotoxicity during high-multiplicity infections, whereas *D. discoideum* grown in suspension was relatively resistant to cytotoxicity and did not support intracellular growth. Some known *D. discoideum* mutants were examined for their effect on growth of *L. pneumophila*. The coronin mutant and the *myoA*/*B* double myosin I mutant were more permissive than wild-type strains for intracellular growth. Growth of *L. pneumophila* in a G_{β} mutant was slightly reduced compared to the parent strain. This work demonstrates the usefulness of the *L. pneumophila-D. discoideum* system for genetic analysis of host-pathogen interactions.

Bacterial pathogenesis involves the interaction of a bacterium with a complex host. Elaborate mechanisms have evolved in microorganisms to manipulate and interfere with host cell functions, and numerous host defenses have arisen to keep pathogens at bay. Recently, there has been interest in studying host-pathogen interactions by using simple, genetically manipulatable hosts. It is hoped that the bacterial factors and host genes involved in causing pathogenic effects in these simple organisms will be relevant to mammalian disease processes. Studies of the expression of antimicrobial peptides in *Drosophila melanogaster* led to the discovery of Toll receptors, critical components of innate immunity that have been recently recognized in mammals (26, 27). Studies of the extracellular bacterial pathogen *Pseudomonas aeruginosa* and its interaction with the worm *Caenorhabditis elegans* indicate that many of the bacterial factors that affect pathogenesis of the worm also affect pathogenesis in the mammalian mouse model and in plants (47). In this report, we introduce the free-living unicellular organism *Dictyostelium discoideum* as a genetically manipulatable host for the intracellular bacterial pathogen *Legionella pneumophila*.

L. pneumophila, the causative agent of Legionnaires' disease, is a gram-negative bacterium that exists as an intracellular parasite of freshwater amoebae (16). Pathogenesis of the bacterium within mammalian hosts and its ability to grow within amoebae are closely linked. In human pneumonia, the microorganism grows in alveolar macrophages, cells that are phagocytic and motile like amoebae (8, 23). Furthermore, *L. pneumophila* mutants defective for growth in macrophages also show defective growth in amoebae (18, 43).

After phagocytosis, *L. pneumophila* is found in a membrane-bound phagosome that avoids fusion with endocytic and lysosomal compartments and is not acidified (21, 22). Examination of markers from the endocytic pathway on the *L. pneumophila* phagosome indicate that avoidance of the endocytic pathway occurs within 10 min of uptake (40, 56). A defining feature of the *L. pneumophila* phagosome in macrophages is its association with ribosomes thought to be derived from rough endoplasmic reticulum (RER) (20, 45). As the infection proceeds, the bacterium-laden phagosome grows until it nearly fills the cell (20, 45). Cell lysis or apoptotic death releases the bacteria to initiate another round of infection (34). *L. pneumophila* can also kill cells by a different mechanism called contact-dependent cytotoxicity (25). At relatively high multiplicities of infection (MOI), contact between the bacteria and cells can cause osmotic lysis of the cells. No internalization of the bacteria is necessary for cytotoxicity, and the link between intracellular growth and cytotoxicity remains unclear.

Genetic hunts have identified approximately 24 *L. pneumophila* genes required for intracellular growth, many of which are also required for contact-dependent cytotoxicity. These genes have been named *dot*/*icm* genes (1, 3, 5, 38, 41, 42, 53). Many of the *dot*/*icm* genes are homologous to genes required for mobilization of conjugal plasmids, and indeed the *dot*/*icm* gene products are required for conjugal transfer of RSF1010 plasmids from *L. pneumophila* (41, 53). This has led to the hypothesis that the *dot*/*icm* gene products form a transport system that is thought to aid pathogenesis not by transferring DNA but by transporting an as yet unidentified effector protein(s) into the host cell. Determining the exact functions of the *dot*/*icm* genes and identifying the transported effector molecules remains a major challenge.

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D. discoideum is a unicellular, free-living organism that lives in soil and feeds on bacteria (4). In the amoebal form, the cells are highly motile and are very active in phagocytosis. A body of literature describes the endolysosomal and phagosomal pathways in *D. discoideum* (references 6, 31, and 39 and references therein). During starvation, the organism undergoes a complex developmental cycle in which the normally free-living single cells aggregate to form a multicellular organism, a motile, phototactic slug. The slug further develops into a fruiting body containing *D. discoideum* spores and a stalk (4, 28, 49). The axenic strains of *D. discoideum* that are routinely used are easily maintained and can grow in pure culture in a rich medium in the absence of bacteria (44, 57).

The availability of genetic tools makes *D. discoideum* a genetically tractable host organism for analysis of host-pathogen interactions. The organism is haploid and has a relatively small genome of 34 Mb (10, 30). It is possible to transform the cells by electroporation and to knock out genes by homologous recombination and marker replacement (12, 32). There are plasmids that replicate in *D. discoideum* that can be used for complementation or ectopic expression (32). There is extensive DNA sequence information available (http://dicty.cmb.nwu.edu/dicty/dicyostelium_genomics.htm); and the complete genome sequence should be finished by 2002.

This report describes the establishment of conditions for the intracellular growth of *L. pneumophila* in *D. discoideum*. Genetic and cell biological analyses indicate that the mechanism of growth in *D. discoideum* is similar to that observed in macrophages and amoebae.

MATERIALS AND METHODS

Cells, strains, and routine maintenance. *D. discoideum* AX3 was a kind gift from D. Knecht (University of Connecticut, Storrs) and was used in all experiments except for analysis of growth of *L. pneumophila* in *D. discoideum* mutants (29). The G_β mutant (strain LW6) and its parent strain DH1 (58) were a kind gift from P. Devreotes (Johns Hopkins University, Baltimore, Md.). The coronin mutant (strain HG1569) and its parent strain AX2-214 (11) were kind gifts from M. Maniak (MRC-LMCB, London, England). The myosin I *myoA* (clone HTD2-4), *myoB* (clone HTD4-3), and *myoA/B* (clone HTD5-4) (36, 50, 55) mutants and their parent strain KAX3 were kind gifts from M. Titus (University of Minnesota, Minneapolis). Other strains examined include the strain overexpressing constitutively active *rab7* (AX4 with pRab7) and the control strain carrying the vector alone (AX4 with pDA80-HA) (6) and the Δ*Ddpik1* Δ*Ddpik2* phosphatidylinositol double 3-kinase mutant strain (59).

Cells grown axenically were cultured in HL-5 liquid medium (44) supplemented with penicillin and streptomycin (100 U/ml; GibcoBRL) and other supplements (Curacil [20 μg/ml] and G418 [7.5 to 20 μg/ml]) as needed. *D. discoideum* was also grown as plaques on a lawn of *Klebsiella aerogenes* plated on SM/5 agar medium (44).

The *L. pneumophila* Benidorm (030E) strain was a kind gift from B. Fields (Centers for Disease Control and Prevention, Atlanta, Ga.); the *L. pneumophila* Philadelphia-1 strain was also obtained from the Centers for Disease Control and Prevention. All genetically manipulated strains are derived from *L. pneumophila* Philadelphia-1. Strain Lp01 is proficient for intracellular growth, streptomycin resistant, and restriction defective (2). Lp01 is the parent of strain HL056, which contains an in-frame deletion of *dotI*, and of strains HL1400 (*dotO*) and HL1700 (*dotH*), which have ethyl methanesulfonate-induced mutations in *dotO* and *dotH*, respectively (1). Strains MW49 and MW50 are derivatives of strains Lp01 and HL056 that express the green fluorescent protein (GFP; expressed by plasmid GFPmut3) from the P_{tac} isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. The strains carry plasmid pAM239, which was constructed by moving the 750-bp *Xba*I-*Pst*I fragment from the GFPmut3 plasmid (9) into the *Xba*I and *Pst*I-digested pMMB207 backbone (33). Strain Lp03 carries a spontaneous mutation in *dotA* (3), strain JV302 carries a spontaneous mutation in *dotB* (54), strain JV328 carries a spontaneous mutation *dotE* (J. Vogel, unpublished results), strain 25D carries a spontaneous mutation in *icmVWX* (5), and strain JV573 carries a spontaneous mutation in *dotG* (53). *L. pneumophila* was grown on plates containing charcoal yeast extract agar (CYE) (14) buffered with ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid; Sigma] to pH 6.9. The same medium without agar and charcoal (AYE) was used for growth of *L. pneumophila* in liquid culture. *K. aerogenes*, a kind gift from D. Knecht, was routinely cultured in Luria broth (LB).

Growth of *L. pneumophila* in *D. discoideum* in liquid culture. *D. discoideum* grown exponentially in shaken flasks in axenic medium was harvested and washed in phosphate-buffered saline (PBS) as follows. Cells were pelleted by a 5 min spin at 600 × *g*, the medium was aspirated and replaced with an equal volume of PBS, the cells were pelleted again by a 5-min spin at 600 × *g*, and the PBS was aspirated. The cells were resuspended in MB medium {20 mM MES [2(*N*-morpholino)ethanesulfonic acid) (pH 6.9), 0.7% yeast extract, 1.4% BBL thiotone E peptone} at 10^6 cells/ml. MB medium is modified HL-5 axenic medium, with glucose omitted and the buffer changed to 20 mM MES, pH 6.9. *L. pneumophila* was harvested after 48 h growth on CYE plates, resuspended in water or PBS, and used to infect *D. discoideum* at an MOI of approximately 1:1. The approximate concentration of bacteria was determined by assuming that A_{600} = 1.0 is equivalent to 10^9 bacteria/ml. Infected cells were distributed to 24-well tissue culture dishes, wrapped in Parafilm to prevent desiccation, and incubated at 25.5°C.

The number of viable *L. pneumophila* or *D. discoideum* on each day was determined by measuring CFU or PFU, respectively. A dilution series of harvested *D. discoideum* was prepared in PBS and plated on lawns of *K. aerogenes* spread on SM/5 medium. The plates were incubated at 21°C, and plaques were counted 3 to 4 days after plating. For quantitation of *L. pneumophila*, a dilution series was prepared from infected *D. discoideum* and plated on CYE plates. *D. discoideum* was lysed to release the intracellular bacteria, either by vigorous vortexing in water or by addition of 0.02% saponin (Sigma S-4521) to the well before harvesting. CYE plates were then incubated at 37°C for 3 or 4 days before colonies were counted.

Transmission electron microscopy. *D. discoideum* was infected with *L. pneumophila* Philadelphia-1 at an MOI of 1:1 in liquid culture as described above. On day 3, infected cells were fixed for electron microscopy using a protocol modified from one previously described (13). Cells were harvested by pipetting up and down, pelleted for 5 min at 600 × *g* at 4°C, resuspended in fixative containing Triton X-100 (0.1 M sodium cacodylate buffer [pH 7.4], 0.01% Triton X-100, 0.5% glutaraldehyde), and incubated for 15 min on ice. Cells were pelleted for 5 min at 600 × *g*, washed in 0.1 M sodium cacodylate buffer (pH 7.4), pelleted again for 5 min at 600 × *g*, and resuspended to single cells in osmium fix (1% OsO_4, 0.1 M sodium cacodylate buffer, pH 7.4) for 30 min at room temperature. The cells were washed twice more with sodium cacodylate (pH 7.4), pelleted, dehydrated in a graded series of alcohols, infused with propylene oxide, embedded in Epon 812, and sectioned into 90-nm slices. Samples were analyzed on a Philips CM-10 transmission electron microscope.

Quantitation of viable bacteria internalized by *D. discoideum*. *D. discoideum* grown exponentially in HL-5 medium in shaken flasks was harvested, washed in PBS as described above, resuspended in MB medium at 2 × 10^6 cells/ml, plated in 24-well tissue culture dishes, and incubated at 25.5°C. *L. pneumophila* was grown at 37°C in AYE liquid medium to an A_{600} of 3 to 3.5, to allow maximal infectivity (7), and *K. aerogenes* was grown in LB until stationary phase. Prior to infections, bacterial strains were pelleted for 5 min at 16,000 × *g* in a microcentrifuge and resuspended in MB medium.

D. discoideum was infected with bacteria at an MOI of 5:1, and the infection was initiated by a 5-min spin at 200 × *g*. Thirty minutes after the initiation of infection, gentamicin was added to the medium to a final concentration of 50 μg/ml, to kill noninternalized bacteria, and was maintained in the medium for the duration of the experiment. At various times after infection, cells were washed two times with PBS to remove gentamicin and lysed with 0.02% saponin. A dilution series of the harvested bacteria was prepared in PBS prior to plating for CFU on CYE medium (*L. pneumophila*) or LB agar (*K. aerogenes*).

Immunofluoresence. *D. discoideum* grown exponentially in axenic medium in shaken flasks was harvested, washed in PBS as described above, and resuspended in MB with 2 mM IPTG at 10^6 cells/ml. Cells were plated on poly-L-lysine-coated coverslips in tissue culture wells and allowed to adhere and equilibrate at 25.5°C for at least 1 h before bacteria were added. *L. pneumophila dot*$^+$ and *dotI* strains expressing GFP were grown to optimal infectivity in AYE–2 mM IPTG (A_{600} = 3 to 3.5), pelleted, and resuspended in MB–2 mM IPTG medium. *D. discoideum* was infected with bacteria at an MOI of 10:1, and the infection was initiated with a 5 min spin at 200 × *g*.

Thirty minutes after infection, cells were fixed in 2% paraformaldehyde in PBS with one-third-strength HL-5 medium and 0.1% dimethyl sulfoxide for 5 min at room temperature (6). Samples were permeabilized by incubation in methanol containing 1% paraformaldehyde for 5 min at −20°C, washed at least five times in PBS, and blocked in 4% goat serum (Gibco-BRL)–PBS for 1 h. Antibodies for staining were diluted in PBS containing 2.5 mg of bovine serum albumin per ml and 0.1% saponin. The V-ATPase antigen was visualized using a 1:20 dilution of a monoclonal antibody against the 100-kDa subunit (17), and lysosomal membranes were visualized using a 1:1,000 dilution of a polyclonal antibody generated against purified lysosomal membrane proteins from *D. discoideum*. After 1-h incubations with the primary antibodies, the samples were washed 5 times in PBS and secondary antibodies coupled to Texas red were added at a 1:500 dilution (Molecular Probes). Fixed samples were analyzed using a Nikon TE300 microscope, and images were captured by a Princeton Micromax slow-scan cooled charge-coupled device camera. To raise serum against lysosomal membrane proteins, the fraction was purified as described elsewhere (48) and injected intramuscularly without adjuvant into rabbits. The primary injection of 100 μg was followed by two boosts of 100 μg each, and serum was collected after the second boost.

Cytotoxicity assay. *D. discoideum* grown exponentially in axenic medium in shaken flasks was harvested, washed in PBS as described above, and resuspended in MB medium at 10^6 cells/ml. Cells were incubated either as adherent monolayers in tissue culture wells or in suspension in silanized 50-ml conical tubes shaken in a water bath. Cells were equilibrated for at least 2 h at 25.5°C before bacteria were added. *L. pneumophila* Philadelphia-1 was grown to optimal infectivity in AYE (A_{600} = 3 to 3.5), pelleted, and resuspended in MB medium.

D. discoideum was infected with bacteria at an MOI of 375:1 by adding bacteria in 1/10 the volume of the *D. discoideum* culture and incubating at 25.5°C for approximately 24 h. At the end of the infection, a dilution series of harvested cells was prepared in PBS and plated for PFU on *K. aerogenes*.

Survival of internalized bacteria in adherent or suspended *D. discoideum*. *D. discoideum* grown exponentially in axenic medium in shaken flasks was harvested, washed in PBS as described above, and resuspended in MB medium. One group of cells was plated in tissue culture wells at 2×10^6 cells/ml to form adherent monolayers, while a second group was incubated with shaking in silanized 50-ml conical tubes at 5×10^6 cells/ml. All cells were incubated at 25.5°C and were allowed to equilibrate for approximately 4 h before addition of bacteria. *L. pneumophila* was grown to optimal infectivity in AYE to an A_{600} of 3 to 3.5, and *K. aerogenes* was grown to stationary phase in LB.

Adherent cells were infected with bacteria at an MOI of 1:1, and the infection was initiated with a 5-min spin at $200 \times g$. Cells in suspension were infected at an MOI of 20:1. Thirty minutes after the initiation of infection, gentamicin was added to the medium to a final concentration of 50 μg/ml to kill noninternalized bacteria. The gentamicin remained in the medium for the duration of the experiment. One hour and 19 h after infection, the number of viable intracellular bacteria was determined.

Infected cells were harvested, pelleted for 5 min at $5{,}000 \times g$, washed in PBS to remove gentamicin, pelleted again for 5 min at $5{,}000 \times g$, resuspended in PBS, and lysed with 0.02% saponin. A dilution series of the harvested bacteria was prepared in PBS and plated for CFU.

Growth of *D. discoideum* on lawns of *L. pneumophila*. Lawns of *L. pneumophila* were prepared by spreading Lp01 (*dot*$^+$) or HL056 (Δ*dotI*) on CYE plates containing reduced levels of L-cysteine (0.05 g/liter) and $Fe(NO_3)_3 \cdot 9H_2O$ (0.034 g/liter). Levels of cysteine normally found in CYE plates inhibit growth of *D. discoideum* (data not shown). Bacterial lawns were grown at 37°C for 2 days prior to inoculation of *D. discoideum*. *D. discoideum* cells growing exponentially in axenic medium were harvested, washed, and resuspended in PBS at 10^6 cells/ml; 10^5 washed cells (100 μl) were spotted onto the lawns, and plates were incubated at 21°C for several days.

RESULTS

Growth of *L. pneumophila* in *D. discoideum* in liquid culture. A system was established in which *L. pneumophila* could grow in *D. discoideum* in liquid culture in a manner analogous to intracellular growth of *L. pneumophila* within macrophages. *D. discoideum* will not survive above 27°C, and most clinical isolates of *L. pneumophila* grow best at 37°C. For this reason, initial experiments were performed with *L. pneumophila* Benidorm-1 (030E), which grows well at 25°C. The temperature of incubation, growth medium, and MOI were adjusted. Once optimal growth conditions were established, all further experiments were performed with the better-characterized *L. pneumophila* Philadelphia-1 strain.

D. discoideum was plated as an adherent monolayer in tissue culture dishes and infected by adding *L. pneumophila* to the medium. Over a 4-day period, *L. pneumophila* grew more than 100-fold in the presence of *D. discoideum* (Fig. 1). The rate of growth between days 1 and 2 was rapid, with a doubling time of approximately 6 h, but slowed on days 2 to 4 post infection to a doubling time of approximately 16 h. The growth of *L. pneumophila* depended on the presence of *D. discoideum* in the medium. *L. pneumophila* plated in the medium alone, without *D. discoideum*, did not grow and viability usually decreased over the course of the experiment (Fig. 1). *L. pneumophila* growth in the presence of *D. discoideum* was not caused by feeding on *D. discoideum* corpses because heat-killed *D. discoideum* did not support growth of *L. pneumophila* (data not shown). It is also clear that live *D. discoideum* was not cross-feeding *L. pneumophila* because if bacteria and live cells were separated by a 0.4-μm-pore-size filter, there was no growth of *L. pneumophila* (data not shown). *D. discoideum* plated in the absence of bacteria remained viable over the course of the experiment but did not grow using these assay conditions (Fig. 1). In the presence of *L. pneumophila*, the number of viable *D. discoideum* remained unchanged until day 2 but then dropped rapidly on days 3 and 4 postinfection (Fig. 1). One likely explanation is that only a small fraction of the *D. discoideum* organisms are initially infected, and so no detectable drop in viability of the *D. discoideum* was seen. Two days postinfection, the titer of bacteria had increased sufficiently due to intracellular growth to allow killing of *D. discoideum* by a combination of cytotoxicity and continued intracellular growth.

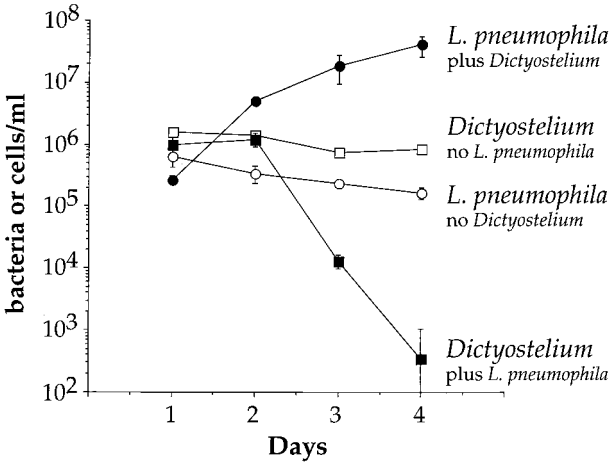

FIG. 1. Growth of *L. pneumophila* in the presence of *D. discoideum* in liquid culture. *D. discoideum* was plated into tissue culture wells in MB medium. Cells were infected with wild-type *L. pneumophila* Philadelphia-1 at an MOI of 1:1. *L. pneumophila* and *D. discoideum* were counted by measuring CFU and PFU, respectively. The experiment was performed twice, each point in the experiment was done in triplicate, and the error bars indicate $n - 1$ weighted sample standard deviation.

Growth of *L. pneumophila* in *D. discoideum* is intracellular. To determine whether the growth of *L. pneumophila* in the presence of *D. discoideum* was occurring intracellularly, a culture infected for 72 h was examined by electron microscopy (Fig. 2). Bacteria were uniformly found in membrane-bound vacuoles within *D. discoideum*. Every stage of intracellular growth could be found, including phagosomes having single cells (Fig. 2D), a bacterium apparently in the process of dividing within a phagosome (Fig. 2A), vacuoles containing a few bacteria (Fig. 2B), and cells nearly taken over by their bacterium-filled phagosomes (Fig. 2C). Association with RER is a defining feature of the *L. pneumophila* phagosome in macrophages (20, 45). In *D. discoideum*, RER can be seen associated with the phagosomes either in one layer (Fig. 2A) or multiple layers (Fig. 2D). In macrophages, the *L. pneumophila* phagosomal membrane was sometimes lined with ribosomes, a phenomenon that can also be seen in *D. discoideum* (Fig. 2B). These micrographs show that the *L. pneumophila* phagosomes in *D. discoideum* have the same characteristic association with ribosomes as seen in macrophages.

Growth of *L. pneumophila* in *D. discoideum* depends on *dot* gene functions. The *dot* genes of *L. pneumophila* are essential for establishing intracellular growth of *L. pneumophila* in macrophages and amoebae (18, 43). The analysis of growth of wild-type and three isogenic *dot* mutant strains of *L. pneumophila* in *D. discoideum* indicates that intracellular growth similarly requires the products of multiple *dot* loci (Fig. 3). The wild-type bacteria showed a characteristic 100-fold growth, while *dotH*, *dotI*, and *dotO* mutants all failed to grow and lost viability over the course of 4 days (Fig. 3).

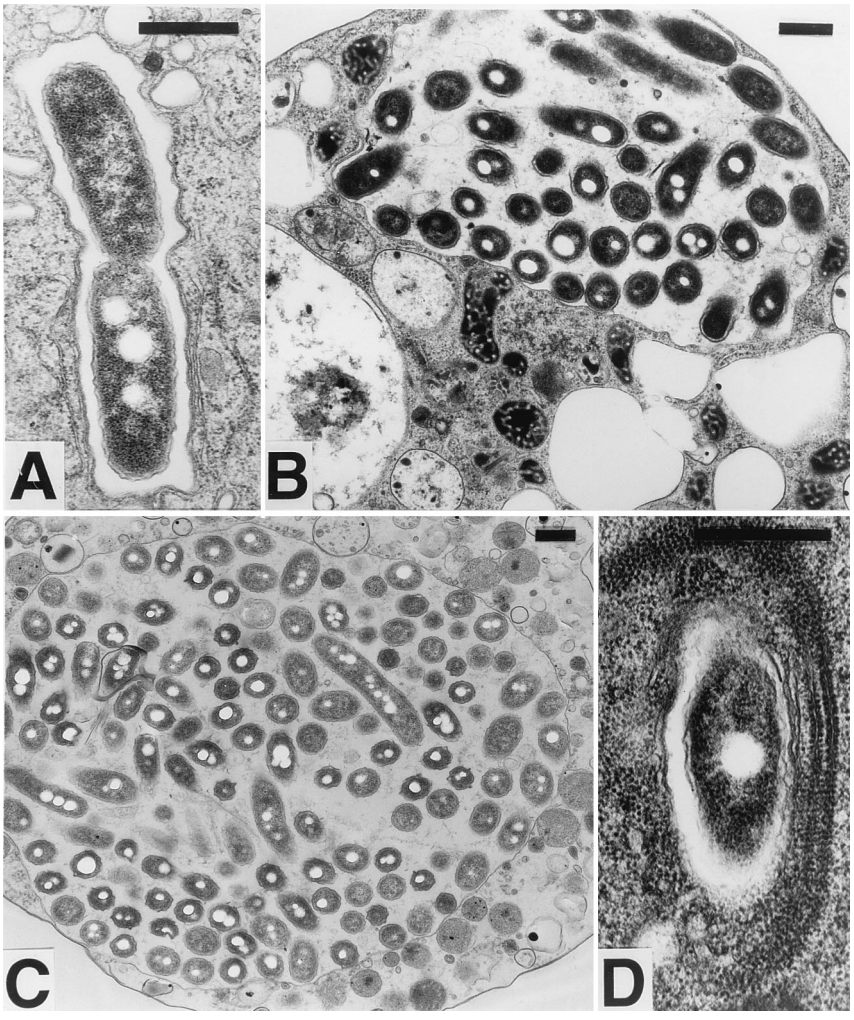

FIG. 2. Transmission electron microscopy of *D. discoideum* infected with *L. pneumophila*. *D. discoideum* was infected with *L. pneumophila* Philadelphia-1 as in Fig. 1. On day 3, cells were harvested and prepared for electron microscopy. (A) Phagosome containing a bacterium apparently in the process of dividing; (B) vacuole containing a few bacteria; (C) a cell nearly taken over by a bacterium-filled phagosome; (D) multiple layers of RER associated with an *L. pneumophila* phagosome. Association of ribosomes with phagosomes can also be seen in panels A and B. In all panels, the bar equals 0.5 μm.

Wild-type *L. pneumophila* avoids killing within *D. discoideum*. The fate of bacteria internalized by *D. discoideum* was followed carefully in the first few hours after infection (Fig. 4). Cells were infected with bacteria; after 30 min, gentamicin was added to the medium to kill all extracellular bacteria and remained in the medium for the duration of the experiment. Internalized *K. aerogenes* was rapidly killed, with the number of viable bacteria dropping over 2 logs in 3 h; the Δ*dotI* mutant bacteria exhibited a similar fate (Fig. 4). Interestingly, intracellular wild-type *L. pneumophila* persisted at the same level of viability over the course of the experiment, indicating that the bacteria resisted digestion (Fig. 4). This experiment, however, does not distinguish whether the internalized wild-type *L. pneumophila* avoids fusion with endocytic compartments or survives within a fused phagolysosome.

Colocalization of internalized *L. pneumophila* and lysosomal membrane proteins. The association of internalized *L. pneumophila* with endosomes and lysosomes was examined by immunofluorescence. After 30 min of infection, *L. pneumophila* internalized by *D. discoideum* did not colocalize with a monoclonal antibody staining the V-ATPase (17) (data not shown). An antibody was generated against purified lysosomal membrane proteins (Materials and Methods). The antibody stained the plasma membrane and faintly stained numerous vesicles within the cell. Thirty minutes after infection, 6% (12 of 202) of *dotI* mutant bacteria were colocalized with anti-lysosomal membrane protein staining. Positive scoring was based on seeing large rings of lysosomal membrane protein staining around these *dotI* mutant bacteria (Fig. 5C and F). In contrast, none of 62 *L. pneumophila dot*$^+$ bacteria colocalized with the lysosomal membrane protein staining (Fig. 5I).

***D. discoideum* grown in suspension is resistant to *L. pneumophila* cytotoxicity and intracellular growth.** In all of the growth experiments described above, *D. discoideum* was infected as adherent cells in a monolayer. Traditionally, phagocytosis has been measured in *D. discoideum* suspended in shaking culture because shaking is thought to reduce the nonspecific interactions between particles and cells (52). In attempts to measure phagocytosis of *L. pneumophila* by *D. discoideum* in suspension, we observed that the amoebae were resistant to *L. pneumophila*.

Contact-dependent cytotoxicity was greatly reduced in *D. discoideum* grown in suspension (Fig. 6). If adherent *D. discoideum* cells in MB medium were exposed to a high MOI (375:1)

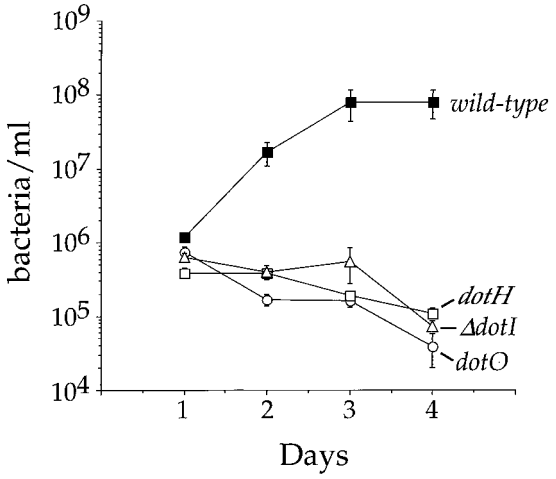

FIG. 3. Growth of wild-type and isogenic *dot* mutant *L. pneumophila* in *D. discoideum*. *D. discoideum* was plated in tissue culture wells in MB medium and infected with *L. pneumophila* at an MOI of 1:1. The number of viable bacteria was determined by counting CFU. The experiment was performed three times, each point in the experiment was done in triplicate, and the error bars indicate the $n - 1$ weighted sample standard deviation.

of wild-type *L. pneumophila*, the number of viable *D. discoideum* was reduced by 10^4 in 24 h (Fig. 6, adherent cells). We attribute this rapid decline in viability to contact-dependent cytotoxicity, as there was no detectable growth of *L. pneumophila* (data not shown). In contrast, suspended *D. discoideum* cells in MB medium exposed to the same multiplicity of wild-type *L. pneumophila* lost only fivefold viability after 24 h (Fig. 6, suspended cells). This fivefold drop in viability was larger than the sample standard deviations and is therefore statistically significant.

A potential explanation for this phenomenon is that *D. discoideum* and *L. pneumophila* are unable to interact in shaking culture. However, shortly (1 h) after infection of suspended *D. discoideum*, abundant intracellular *L. pneumophila* bacteria

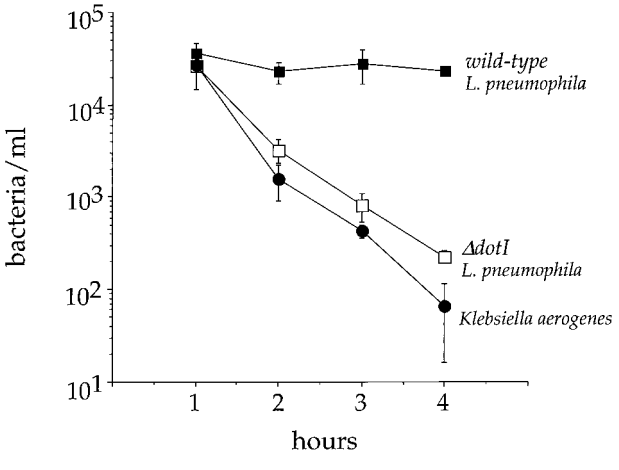

FIG. 4. Viability of bacteria internalized by *D. discoideum* 1 to 4 h after infection. *D. discoideum* was plated in tissue culture wells in MB medium and infected with bacteria at an MOI of 5:1. After 30 min, gentamicin was added to kill extracellular bacteria. The number of viable intracellular bacteria remaining was counted by measuring CFU extracted from washed cells. The experiment was performed two times, each point was done in duplicate, and the error bars indicate the $n - 1$ weighted sample standard deviation.

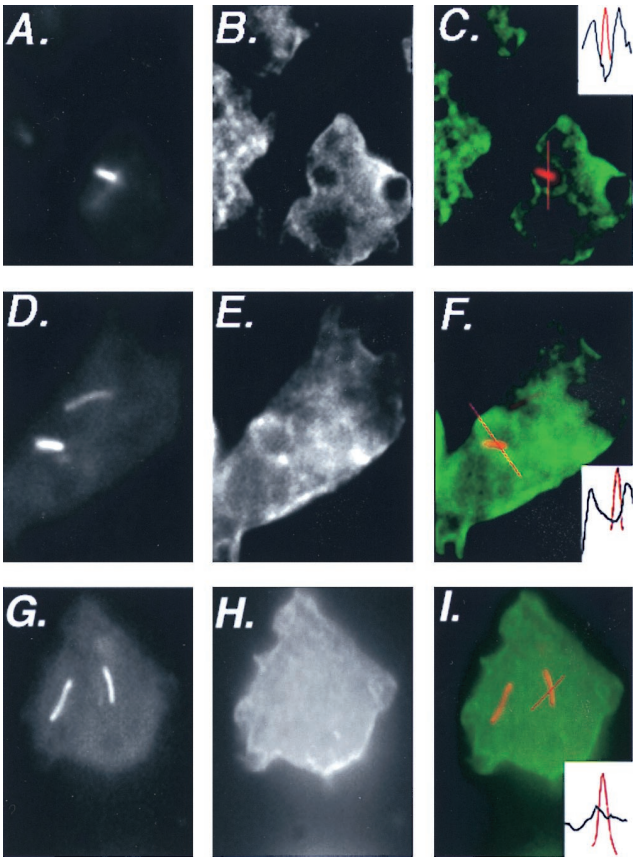

FIG. 5. Association of lysosomal membrane proteins with *L. pneumophila*-containing phagosomes in *D. discoideum*. *D. discoideum* was plated on coverslips in tissue culture wells in MB medium and infected with *L. pneumophila* at an MOI of 10:1. After 30 min of infection, cells were fixed for immunofluoresence analysis. Cells in panels A to C and D to F were infected by *dotI* mutant *L. pneumophila*; cells in panels G to I were infected with *dot*$^+$ *L. pneumophila*. Panels A, D, and G show staining of the bacteria; panels B, E, and H show staining of the anti-lysosomal membrane protein antibody. In panels C, F, and I, the two images are superimposed, with bacterial staining shown in red and lysosomal membrane protein staining shown in green. The inset indicates the bacterial fluorescence (red) or the lysosomal membrane protein fluorescence (black) along the line drawn in each panel. Images were processed with IP Lab Spectrum version 3.2.

were observed by fluorescence microscopy after differential staining of extracellular and intracellular bacteria (data not shown). Bacteria incubated with suspended cells showed significant protection from gentamicin killing, further indicating the bacteria were intracellular (Fig. 7).

Based on gentamicin protection, wild-type *L. pneumophila* was internalized but failed to survive in *D. discoideum* suspended cells (Fig. 7). Adherent cells and suspended cells were infected with *L. pneumophila dotI* and *dot*$^+$ strains, as well as with *K. aerogenes*, and extracellular bacteria were killed 30 min after infection by addition of the antibiotic gentamicin to the medium. Both adherent and suspended *D. discoideum* internalized all three strains of bacteria, as indicated by gentamicin protection 60 min postinfection. As expected, the number of viable internalized *L. pneumophila dotI* and *K. aerogenes* was below the limit of detection after 19 h of infection in both adherent and suspended *D. discoideum*. In contrast, the *L. pneumophila* (*dot*$^+$) in adherent cells were viable at 19 h postinfection. A very different result was observed with suspended cells. *L. pneumophila dot*$^+$ was internalized by sus-

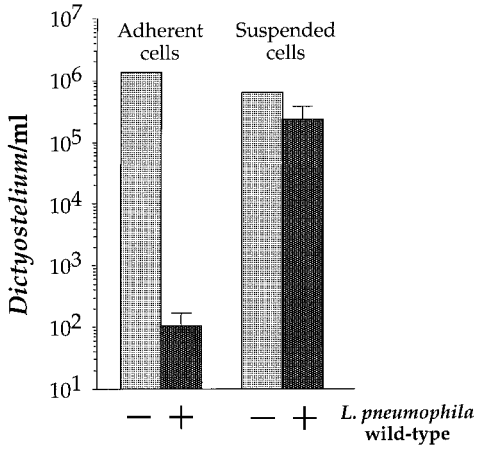

FIG. 6. Susceptibility of adherent and suspended *D. discoideum* to a high-MOI infection of *L. pneumophila*. *D. discoideum* was incubated in MB medium either as adherent monolayers in tissue culture wells or as suspended cells shaken in tubes. Cells were infected (or not) with *L. pneumophila* Philadelphia-1 at an MOI of 375:1 for approximately 24 h. Viable *D. discoideum* cells were counted by measuring PFU. The experiment was performed twice, each condition was done in duplicate, and the error bars indicate the $n - 1$ weighted sample standard deviation.

pended cells and killed by 19 h post infection (Fig. 7). Thus, under conditions used in this assay system, suspended *D. discoideum* was not permissive for *L. pneumophila* growth.

Growth of *D. discoideum* on lawns of *L. pneumophila*. *D. discoideum* is routinely grown on lawns of bacteria, usually *K. aerogenes* (44). Colonies of amoebae form plaques on the lawn after several days. Altered plaque phenotypes have been useful in genetic screens for *D. discoideum* mutants, and so the growth of *D. discoideum* on lawns of *L. pneumophila* (*dot*$^+$ and *dot* mutant) was examined.

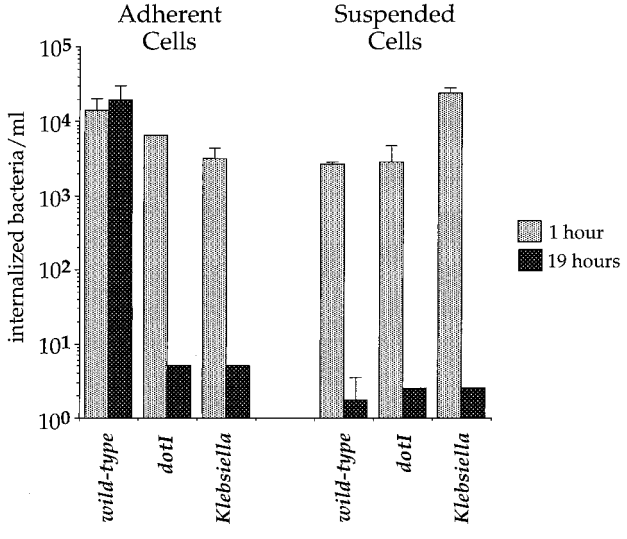

FIG. 7. Survival of internalized bacteria in adherent and suspended *D. discoideum*. *D. discoideum* was incubated in MB medium either as adherent monolayers in tissue culture wells or as suspended cells shaken in tubes. Cells were infected with bacteria for 30 min, at which point gentamicin was added to kill extracellular bacteria. Viable, intracellular bacteria remaining were counted by measuring CFU extracted from washed cells. The experiment was performed twice, each condition was done in duplicate, and the error bars indicate the $n - 1$ weighted sample standard deviation.

D. discoideum was unable to grow on a lawn of *L. pneumophila* (*dot*$^+$) but was able to grow on a lawn of an isogenic *dotI* mutant (Fig. 8). *D. discoideum* spotted onto the lawn of Δ*dotI* *L. pneumophila* made a large clearing in the lawn, and fruiting bodies developed as determined by visual inspection (Fig. 8B). Several *dot* mutants were tested, including strains having mutations in *dotA*, *dotB*, *icmVWX*, *dotE*, *dotG*, *dotH*, and *dotO*, and all were able to support *D. discoideum* growth (data not shown). These results suggest that *D. discoideum* can utilize a variety of *L. pneumophila dot* strains as a food source and that the failure of *D. discoideum* to grow on wild-type *L. pneumophila* is due to functions supplied by the products of multiple *dot* genes.

In contrast, *D. discoideum* cells on the lawn of wild-type *L. pneumophila* were killed rapidly. Microscopic examination indicated that many more *D. discoideum* cells were recovered 20 h postinfection from the lawn of *dotI* mutant than from the *dot*$^+$ lawn. The *D. discoideum* recovered from the *dotI* lawns showed normal morphology with nuclear material and clustered vesicles surrounded by a clear zone of cytoplasm. The *D. discoideum* cells recovered from the *L. pneumophila* (*dot*$^+$) lawns were round, had no obvious internal organization, and were filled with small vesicles (data not shown). The rapid rate of killing, disturbed cell morphology, and the presence of a high MOI suggested that the *D. discoideum* was killed by contact-dependent cytotoxicity, a process that depends on *dot* gene functions.

Effect of known *D. discoideum* mutants on intracellular growth of *L. pneumophila*. To begin to take advantage of *D. discoideum* genetics, a variety of previously characterized mutants showing defects in phagocytosis or membrane trafficking were plated in adherent monolayers and analyzed for the ability to support growth of *L. pneumophila*. A large number of such mutants exist, most of which contain defined lesions in single genes.

A *D. discoideum* strain having an insertion in G_β, a subunit of trimeric G proteins, supported growth of *L. pneumophila* but at a slightly reduced rate (Fig. 9A). *D. discoideum* has only one G_β subunit, and the null mutation analyzed here theoretically eliminates all trimeric G-protein signaling in the cells (58). The *D. discoideum myoA/B* double myosin I mutant and the coronin mutant, on the other hand, supported growth of *L. pneumophila* as well as, if not better, than the wild-type controls (Fig. 9B and C). The myosin I isoforms in *D. discoideum* play important roles in cell motility and endocytosis (51). Coronin is a WD repeat protein that localizes to the moving portions of the cell, and coronin null mutants show defects in motility, cytokinesis, and phagocytosis and pinocytosis in suspension (11, 19, 31). The enhanced growth of wild-type *L. pneumophila* in the coronin mutant was particularly striking, in that 3 days after infection there was routinely a 10-fold-higher yield of *L. pneumophila* than in wild-type *D. discoideum* (Fig. 9C). We tested additional *D. discoideum* mutants that had no effect on *L. pneumophila* growth, including *myoA* and *myoB* single mutants (50, 55), cells overexpressing constitutively active *rab7* (6), and a double phosphatidylinositol 3-kinase mutant (59) (data not shown).

DISCUSSION

The results indicate that growth of *L. pneumophila* in *D. discoideum* occurs by a mechanism that is similar to its growth in amoebae and macrophages. In macrophages at 37°C, the titer of *L. pneumophila* increases 3 to 4 logs in 3 days (2, 5, 53). In adherent *D. discoideum*, using conditions described here, the titer of *L. pneumophila* increased at least 100-fold over 3

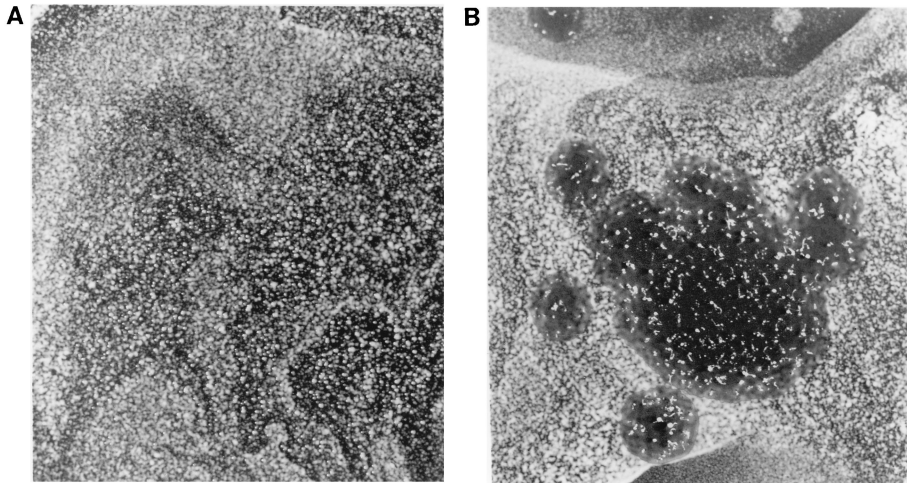

FIG. 8. Growth of *D. discoideum* on lawns of *L. pneumophila* 10^5 *D. discoideum* were spotted onto bacterial lawns grown on CYE plates made with reduced concentrations of cysteine and iron. (A) Lawn of *L. pneumophila* strain Lp01 (*dot*$^+$); (B) lawn of *L. pneumophila* strain HL056 (Δ*dotI*).

days, which is impressive given that the growth temperature was reduced to 25.5°C. *L. pneumophila* did not grow in *D. discoideum* at 21°C (data not shown), indicating that 25.5°C may be close to the lowest temperature that supports *L. pneumophila* intracellular growth.

Growth of *L. pneumophila* occurred within *D. discoideum* in membrane-bound vesicles associated with ribosomes and RER. Under our conditions of fixation, RER associated with *L. pneumophila* phagosomes was more easily detectable in *D. discoideum* compared to macrophages (R. Isberg, personal observation). In some circumstances, such as Fig. 2C, large bacteria-filled vacuoles could be found devoid of ribosomes. This absence of localization may be either a result of extraction of the sample by the detergent present in the fixative used here or because RER sequestration about the vacuole dissipates as the intracellular growth cycle proceeds.

Growth of *L. pneumophila* in *D. discoideum* is dependent on functions provided by multiple *dot* gene products. Three representative *dot* mutants were tested in our experiments, and all were blocked for intracellular growth. A similar phenotype is observed for these same strains in macrophages (1). This result is strong supporting evidence that the intracellular growth observed is initiated in a fashion similar to that seen in macrophages.

L. pneumophila dot$^+$ persisted after internalization by *D. discoideum*, whereas a Δ*dotI* mutant and a *K. aerogenes* control strain were efficiently killed by 4 h (Fig. 4). In cultured macrophages, internalized *dot* mutants fail to grow but remain viable for several days in spite of fusion with lysosomes (46). This difference likely reflects the more effective digestive capabilities of *D. discoideum* relative to cultured macrophages.

We have attempted to directly examine whether phagosomes containing wild-type *L. pneumophila* avoid endosomal fusion in *D. discoideum*, using immunofluoresence to localize the vacuolar ATPase. Thirty minutes after infection, the visualized vacuolar ATPase, which marks the contractile vacuole, did not colocalize with either wild-type or *dotI* mutant *L. pneumophila*-containing phagosomes. The speed at which *D. discoideum* internalizes and digests microorganisms may make it difficult to observe the transient colocalization of V-ATPase with the phagosome. Biochemical examination of early phagosomes in *D. discoideum* showed that the V-ATPase is present in these membranes (39). *L. pneumophila* can efficiently repli-

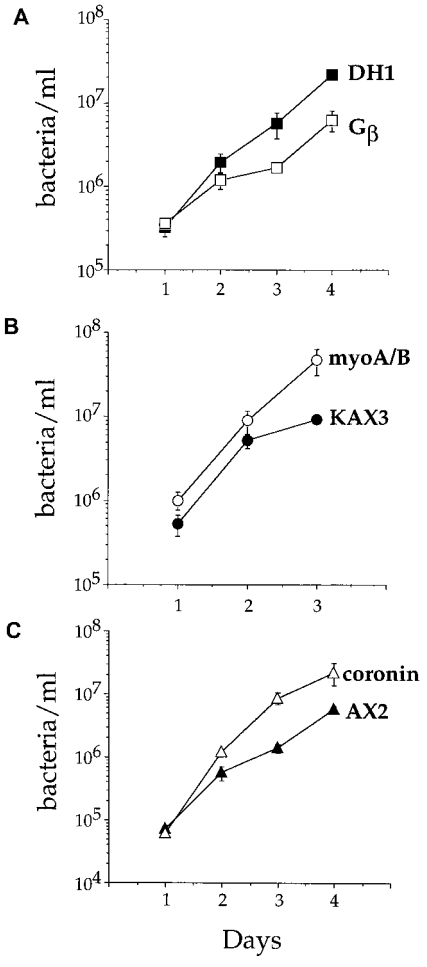

FIG. 9. Growth of *L. pneumophila* in *D. discoideum* mutants. Various *D. discoideum* mutants and their parent strains were plated in tissue culture wells in MB medium and infected with *L. pneumophila* Philadelphia-1 at an MOI of 1:1. The number of viable bacteria was determined by counting CFU. The experiments were performed two to four times depending on the strain, each point in the experiment was done in triplicate, and the error bars indicate the $n - 1$ weighted sample standard deviation.

cate in mammalian cells that lack a contractile vacuole, and so it is not surprising that *L. pneumophila* is not found in that organelle.

The colocalization of intact *L. pneumophila* and lysosomal membrane proteins was also examined. The frequency of clear colocalization was far greater with the *dotI* mutant strain than with *dot*$^+$ bacteria, suggesting that *dot*$^+$ *L. pneumophila* successfully evaded lysosomal fusion in *D. discoideum*. One hour after infection, there was clear microscopic evidence of degradation of a *dot*$^+$ strain, suggesting that evasion of the endocytic pathway may be less efficient in *D. discoideum* than in macrophages (data not shown).

Interestingly, *D. discoideum* grown in suspension was both resistant to cytotoxicity induced by high-MOI infection of *L. pneumophila* and unable to support intracellular growth. The explanation for these observations could be due to either bacterial or host factors. It is possible that tight adherence of bacteria to the target cell is needed for effector proteins to be transferred through the Dot-Icm complex and promote intracellular growth. Shaking of the *D. discoideum* culture itself could disrupt this process in a fashion similar to what is observed when conjugal DNA transfer is disrupted in shaking cultures (24). Increasing the adherence of the bacteria may overcome this block. Alternatively, *D. discoideum* grown in suspension could be limiting for some crucial cellular protein that is a target for a translocated *L. pneumophila* protein, or the suspended amoebae could lack a particular uptake pathway that *L. pneumophila* requires to establish its replicative compartment. Furthermore, *D. discoideum* in suspension may be in an altered state, similar to macrophages activated by treatment with gamma interferon, which results in resistance to *L. pneumophila* infection (35).

All of the *D. discoideum* mutants tested were capable of supporting *L. pneumophila* growth. *L. pneumophila* grew well in both independently derived axenic strains, AX3 and AX2, indicating that *L. pneumophila* growth does not depend on a particular strain of *D. discoideum*. The G_β mutant alone showed a slight reduction in growth of *L. pneumophila*. Previously it had been shown that adherent G_β mutant cells are impaired in phagocytosis as determined by uptake of yeast particles (37) and the fact that they form small plaques on lawns of *K. aerogenes* (58). In this strain, particle attachment is normal, but fewer attached particles are engulfed compared to wild-type cells (37). The slightly reduced growth of *L. pneumophila* in this mutant could be explained by a reduced efficiency of uptake, both for the initial infection and when bacteria reinfect cells after completing a round of intracellular infection.

A recent report suggests that phagosomes bearing *Mycobacterium tuberculosis* are blocked for entry into the endocytic pathway by failing to release a coronin homologue that coats its surface (15). In this model, the presence of this molecule, called TACO, interferes with the ability of this phagosome to traffic into a degradative pathway. Clearly coronin does not play this role for *L. pneumophila* in *D. discoideum*, as the absence of coronin does not prevent *L. pneumophila* growth.

The *myoA*/*B* myosin I double mutant and the coronin mutant allowed better growth of *L. pneumophila* than the parental controls. As adherent cells, the *myoA*/*B* mutant shows no defect in pinocytosis or phagocytosis (36). The coronin mutant shows both pinocytosis and phagocytosis defects in suspension but has not been tested in adherent cells (19, 31). Both of these mutants are defective for amoebal motility. Coronin mutants move at speeds roughly one-third that of wild-type cells (11), and *myoA*/*B* double mutants move at speeds roughly one-half that of wild-type cells (51). Perhaps this gives the *L. pneumophila* growth pathway a kinetic advantage relative to the digestive pathway. By this model, establishment of the *L. pneumophila* replication vacuole is dependent on successful competition of factors produced by the organism that support intracellular growth relative to host cell factors that target the phagosome into a route that prevents replication of the bacterium.

Using the *L. pneumophila-D. discoideum* system, we have begun a genetic analysis of bacterial and host functions involved in this host-pathogen interaction. In theory, any intracellular pathogen that can grow at 25°C may be capable of growth in *D. discoideum*. If such systems can be established, it should be possible to identify host mutants that no longer support growth of almost any pathogen. The products of these host genes may be ideal candidates for drug therapy as it may be possible to mimic the effect of the mutations with small molecules that block intracellular growth of pathogens.

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