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Comparative Analysis of *Legionella pneumophila* and *Legionella micdadei* Virulence Traits

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While the majority of Legionnaire's disease has been attributed to *Legionella pneumophila*, *Legionella micdadei* can cause a similar infection in immunocompromised people. Consistent with its epidemiological profile, the growth of *L. micdadei* in cultured macrophages is less robust than that of *L. pneumophila*. To identify those features of the *Legionella* spp. which are correlated to efficient growth in macrophages, two approaches were taken. First, a phenotypic analysis compared four clinical isolates of *L. micdadei* to one well-characterized strain of *L. pneumophila*. Seven traits previously correlated with the virulence of *L. pneumophila* were evaluated: infection and replication in cultured macrophages, evasion of phagosome-lysosome fusion, contact-dependent cytotoxicity, sodium sensitivity, osmotic resistance, and conjugal DNA transfer. By nearly every measure, *L. micdadei* appeared less virulent than *L. pneumophila*. The surprising exception was *L. micdadei* 31B, which evaded lysosomes and replicated in macrophages as efficiently as *L. pneumophila*, despite lacking both contact-dependent cytopathicity and regulated sodium sensitivity. Second, in an attempt to identify virulence factors genetically, an *L. pneumophila* genomic library was screened for clones which conferred robust intracellular growth on *L. micdadei*. No such loci were isolated, consistent with the multiple phenotypic differences observed for the two species. Apparently, *L. pneumophila* and *L. micdadei* use distinct strategies to colonize alveolar macrophages, causing Legionnaire's disease.

At the 1976 American Legion convention in Philadelphia, an outbreak of pneumonia, termed Legionnaire's disease, led to the identification of the genus *Legionella* (31, 52). Although more than 40 species of *Legionella* are now known (7), surveillance studies have attributed 80 to 90% of Legionnaire's disease cases to *Legionella pneumophila* (14, 50), and it has been studied in the greatest detail. *L. pneumophila* is an opportunistic pathogen: disease is typically restricted to people with underlying health conditions, including the elderly, cigarette smokers, individuals receiving immunosuppressive therapy, or organ transplant recipients (14, 50). In contrast, healthy individuals associated with outbreaks, recognized by their low titers of *Legionella*-specific antibodies, are often asymptomatic (34).

In nature, legionellae are found in fresh water and soil as parasites of amoebae (11, 28, 65). *L. pneumophila* may adapt to its distinct intracellular and aquatic environments by alternating between a "replicative" and a "virulent" form in response to growth conditions (13). When amino acids and other conditions are favorable, *L. pneumophila* replicates within a host cell vacuole. When amino acids are limiting, the bacteria become cytotoxic, resistant to osmotic stress, motile, sensitive to sodium, and competent to evade phagosome-lysosome fusion (13), traits which likely enable the progeny to survive and to disperse in the environment, then reestablish a protected intracellular replication niche. Many of the traits required by *L. pneumophila* to parasitize amoebae also contribute to its survival and growth in macrophages (18, 33, 72).

Although the molecular mechanisms of *L. pneumophila* pathogenesis remain largely undefined, a number of traits which are correlated with virulence have been described. When aerosols containing *L. pneumophila* are inhaled, alveolar macrophages ingest the bacterium by coiling or conventional phagocytosis (39, 62). Phagosomal bacteria can evade phagosome-lysosome fusion, associate sequentially with mitochondria and the rough endoplasmic reticulum (ER), then replicate to high numbers (37, 38, 77). To establish this replication niche, *L. pneumophila* may secrete virulence factors by a specialized transport apparatus. A number of *dot* and *icm* genes are required by *L. pneumophila* for both intracellular growth in macrophages and conjugal DNA transfer (70, 80), and some of the predicted Dot and Icm proteins resemble components of the *Agrobacterium tumafaciens vir* complex (17), the *Bordetella pertussis ptl* system (84), and the *Helicobacter pylori cag* complex (16), three secretion systems important for virulence. In addition, it has been shown that a type II secretion system contributes to the virulence of *L. pneumophila* (49).

The second most common etiologic agent of Legionnaire's disease is *Legionella micdadei* (27, 50, 63), which infects immunocompromised hosts primarily (27, 47, 55, 56, 64, 68). Consistent with its epidemiology, *L. micdadei* is less virulent than *L. pneumophila* in guinea pig and tissue culture models of infection (29, 83). The genetic basis for the differential virulence of *L. micdadei* and *L. pneumophila* has not been determined. Indeed, very few studies have directly compared the pathogenesis of *L. pneumophila* with that of *L. micdadei*, and the variety of *L. micdadei* clinical isolates examined further complicates interpretation of this literature.

L. micdadei and *L. pneumophila* do share some features associated with virulence but not others. For example, like *L. pneumophila*, *L. micdadei* encodes flagella (6, 35), acid phosphatase (26), Mip (19, 57), and common antigen, or GroEL (5, 61), and both species inhibit superoxide anion generation by macrophages (25, 26). However, *L. micdadei* lacks phospholipase C and zinc metalloprotease, two factors thought to play a role in *L. pneumophila* virulence (26). And unlike *L. pneumophila*, macrophage internalization of *L. micdadei* via

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coiling phagocytosis (62) and association with the host ER (83) has not been observed. Indeed, whether *L. micdadei* belongs in the genus *Legionella* is controversial. As judged by the 16S rRNA similarity (30, 32) and DNA relatedness profiles (10), it can be argued that *L. micdadei* and *L. pneumophila* are distinct taxonomically. In spite of their genetic heterogeneity, some contend that the legionellae form a natural and practical phenotypic genus (7, 10).

A number of quantitative assays have been developed to evaluate particular traits associated with *L. pneumophila* virulence (13, 15, 41, 46, 60, 69, 79, 80, 82). To determine whether robust intracellular growth correlates with expression of one, or several, of these traits, four *L. micdadei* clinical isolates were compared to one well-characterized *L. pneumophila* strain. In addition, using a genetic gain-of-function strategy, we investigated whether any *L. pneumophila* loci could stimulate *L. micdadei* growth in cultured macrophages. Results obtained from both the approaches suggest that *L. pneumophila* and *L. micdadei* employ different strategies to replicate within amoebae and macrophages.

MATERIALS AND METHODS

Cell culture. Bone marrow-derived macrophages were prepared from female A/J mice (Jackson Laboratory) as described previously (77). After a 7-day culture period in L-cell conditioned medium, macrophages were collected by centrifugation, suspended in RPMI 1640 containing 10% fetal bovine serum (RPMI-FBS; Gibco BRL), and plated as described below for intracellular infection and replication, phagosome-lysosome fusion, and cytotoxicity assays. Cells of the human monocyte cell line U937 (American Type Culture Collection) were cultured in RPMI-FBS containing 25 mM HEPES buffer, pH 7.0, and differentiated by treatment with phorbol 12-myristate 13-acetate (Sigma) as described previously (60).

Bacterial strains and media. *L. pneumophila* Lp02, a virulent thymine auxotroph derived from the serogroup 1 Philadelphia-1 strain, was chosen as the prototype because it has been studied extensively (1, 8, 9, 12, 13, 21, 22, 36–38, 40, 46, 66, 67, 69, 70, 73, 74, 77–79). Four clinical isolates of *L. micdadei* were examined: 31B (University of Pittsburgh Hospital), Rivera (Stanford University Medical Center), and Camilleri (Stanford University Medical Center), kindly provided by Nicholas P. Cianciotto, and D-2676 (National Center for Infectious Diseases), kindly provided by Barry S. Fields. Bacterial strains were maintained at −70°C as glycerol stocks. Prior to experiments, strains were colony-purified either on *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal-yeast extract agar (CYE) or on CYE supplemented with 100 μg of thymidine/ml (CYET) and then cultured in ACES-buffered yeast extract broth (AYE) or in AYE supplemented with 100 μg of thymidine/ml (AYET).

Broth cultures of *L. pneumophila* were shown previously to express the following virulence traits exclusively in the stationary phase: sodium sensitivity, osmotic resistance, contact-dependent cytotoxicity, efficient infection initiation, and evasion of phagosome-lysosome fusion (13). Therefore, to learn when *L. micdadei* virulence was maximal, the relationship between growth phase, optical density at 600 nm (OD_{600}), and virulence was determined for each of the *L. micdadei* strains. Entry and survival in macrophages by *L. micdadei* was similar for exponential- and post-exponential-phase cultures (data not shown). However, based upon sodium sensitivity (Na^s) (see Fig. 5) and osmotic sensitivity (see Fig. 7) assays, *L. micdadei* was maximally virulent in the post-exponential phase of growth, which typically began at OD_{600}s of 1.0 for 31B, 1.2 for Camilleri, 1.3 for D-2676, and 1.5 for Rivera (data not shown). Hence, unless stated otherwise, post-exponential-phase cultures were analyzed.

Intracellular bacterial growth. The abilities of *Legionella* strains to enter and survive in macrophages and to replicate within macrophages were measured as described previously (13, 77). Macrophages and U937 cells plated at a density of 2×10^5 to 3×10^5 cells per well in 24-well tissue culture dishes were infected for 2 h; next, extracellular bacteria were removed by washing the monolayers with RPMI-FBS, then incubating with 10 μg of gentamicin/ml in RPMI-FBS for 30 min. Infection initiation efficiency was calculated for triplicate samples by dividing the number of CFU associated with the monolayer at 2 h by the number of CFU added initially to the monolayer, then multiplying by 100. Bacterial replication in mouse macrophages and U937 cells was measured in triplicate over the subsequent 2- or 3-day period. Macrophages were infected with 31B and Lp02 at an approximate multiplicity of infection (MOI) of 1. For Camilleri, Rivera, and D-2676 infections, the MOI was increased to 5 to 10 to ensure that the number of CFU at 2 h was within the range of detection; neither the efficiency of infection initiation nor the growth rate of the strains was affected by the MOI (data not shown).

Microscopic assay for intracellular growth. The ability of individual intracellular bacteria to replicate was analyzed by fluorescence microscopy essentially as described previously (79). Macrophages cultured on 12-mm glass coverslips were infected at approximates MOI of 1 for Lp02 and 31B, 10 for Rivera, 50 for Camilleri, and 25 for D-2676, conditions which ensured that only very rarely were macrophages infected by more than one bacterium. After 2 h, extracellular bacteria were killed by incubating the cultures with gentamicin (10 μg/ml) for 30 min. At this time, one set of coverslips was fixed with periodate-lysine-paraformaldehyde (53) containing 4.5% sucrose and prewarmed to 37°C, washed three times with phosphate-buffered saline (PBS), then stored at 4°C until further use. The remaining coverslips were incubated for an additional 16 h at 37°C to allow Lp02 to replicate within, but not escape from, the primary host cell. The 18-h samples were fixed as described above; then all of the samples were methanol extracted and washed three times with PBS before the DNA was stained fluorescently with 0.1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml of PBS. For each time point, at least 50 infected macrophages were located, and the number of intact bacteria per macrophage was counted.

Phagosome-lysosome fusion assay. The ability of each *Legionella* strain to evade phagosome-lysosome fusion was analyzed 2 h after infection by using fluorescence microscopy and the soluble endocytic probe Texas red-ovalbumin (TRov) as described previously (77). Prior to methanol extraction of the preparations, extracellular bacteria were stained with rabbit serum specific for *L. micdadei* (Monoclonal Technologies, Inc., Norcross, Ga.) or *L. pneumophila* (77) (a kind gift from Ralph R. Isberg) followed by Cascade Blue-conjugated anti-rabbit immunoglobulin G (IgG) antibody (Molecular Probes, Eugene, Oreg.). After methanol extraction of the preparations, both intracellular and extracellular bacteria were stained with *L. micdadei*- or *L. pneumophila*-specific antiserum followed by Oregon Green-conjugated anti-rabbit IgG (Molecular Probes). 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, then multiplying by 100.

Strain 31B stained poorly with the *L. micdadei*-specific antiserum used in this study, making intracellular bacteria difficult to locate. Therefore, an alternate technique was used to label 31B cells fluorescently prior to infection as described before (76). 31B cells were incubated with 5(6)-carboxyfluorescein-*N*-hydrosuccinimide ester (FLUOS; Boehringer Mannheim Biochemica) for 30 min on ice and were washed twice with PBS and once with RPMI-FBS prior to use. FLUOS-labeled bacteria replicated within macrophages as efficiently as untreated cells, as judged by quantifying the yield of CFU from macrophage cultures 24 h after infection (data not shown). Extracellular bacteria were labeled preferentially by incubating the fixed preparations with DAPI, which does not stain phagosomal bacteria brightly when preparations are fixed but not methanol extracted (data not shown).

Cytotoxicity assay. To quantify contact-dependent cytotoxicity, macrophages plated at a density of 3×10^4 to 5×10^4 cells per well of a 96-well tissue culture dish were incubated for 1 h with dilutions of each *Legionella* strain. Next, the infection medium was aspirated, the monolayers were incubated for 4 h with 0.1 ml of 10% (vol/vol) Alamar Blue (Accumed, Inc., Chicago, Ill.) in RPMI-FBS, and the redox-specific absorbance of the dye was measured as the OD_{570} and OD_{600} with a SpectraMax 250 spectrophotometer (Molecular Devices). The percentage of macrophages that were viable was calculated for triplicate samples from the slope of a plot of the A_{570}/A_{600} determined for triplicate samples of six known densities of uninfected macrophages in the range of 10^3 to 5×10^4 cells per well. To determine the actual MOI, duplicate samples of the infection medium were plated onto CYE or CYET.

Sodium sensitivity and osmotic sensitivity. The Na^s of exponential- and post-exponential-phase broth cultures was determined as described previously (13) and calculated as (CFU on CYE or CYET agar containing 100 mM NaCl)/(CFU on CYE or CYET) × 100. The osmotic sensitivity of exponential- and post-exponential-phase broth cultures was determined as described previously (13) by using AYE or AYET broth which did or did not contain 0.3 M KCl and was calculated as (CFU of KCl-treated samples)/(CFU of untreated samples) × 100.

Conjugation efficiency assay. How efficiently each *Legionella* strain donated plasmid DNA by conjugation was quantified by the method of Vogel et al. (80). First, each of the *Legionella* strains was transformed with pMS8 (1), a derivative of the mobilizable IncQ RSF1010 plasmid, which confers kanamycin resistance. Approximately 10^9 CFU of exponential-phase donor cells was mixed with 10^8 to 10^9 CFU of the recipient strain, *Escherichia coli* DH5α. The bacteria were collected by centrifugation, spread onto CYE or CYET, then incubated for 4 h at 37°C. To select for transconjugants, the mating mixture was collected onto a sterile swab, diluted into water, and plated onto Luria broth (LB)-kanamycin, which does not permit *Legionella* growth. Conjugation efficiency was calculated as (CFU of transconjugants)/(CFU of donor) × 100.

Genetic screen for genes that confer increased growth and survival in macrophages on *L. micdadei*. To identify *L. pneumophila* genetic loci which enhance the intracellular growth of *L. micdadei*, we chose strain D-2676 as a cloning vehicle to minimize the background of false-positives. An *L. pneumophila* genomic library (1) consisting of 5- to 10-kb *Sau*3A Lp02 genomic fragments cloned into vector pMS8, which confers kanamycin resistance, was transferred by conjugation to D-2676. Transconjugants were collected as three separate pools, each containing approximately 10^4 members and representing more than 15 genome equivalents. To enrich for *L. micdadei* strains that replicated efficiently intracellularly, each pool of transconjugants was added at an approximate MOI of 1 to cultures of 2×10^6 macrophages in a six-well tissue culture dish. After a

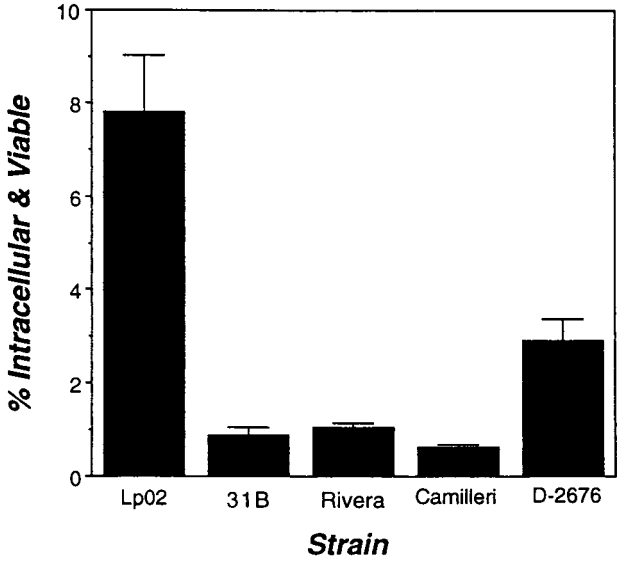

FIG. 1. Efficiency of initiation of infection. Macrophages were incubated with each *Legionella* strain for 2 h; then the numbers of viable and cell-associated bacteria were determined by a standard gentamicin resistance assay (see Materials and Methods). Shown are the means and standard errors of the means calculated in one experiment performed in triplicate; similar results were obtained in six additional experiments.

4.5-h incubation, extracellular bacteria were removed by washing the monolayers with RPMI-FBS, then incubating the infected cultures with 10 μg of gentamicin/ml for 4 h. After 3 days, monolayers were lysed in PBS, and the surviving bacteria were recovered on CYE-kanamycin. Each pool of surviving bacteria was then subjected to a second enrichment cycle.

Next, individual colonies from each enrichment pool were tested for the ability to kill macrophages. For this purpose, clones were cultured to the post-exponential phase in 96-well microtiter dishes containing AYE-kanamycin. An aliquot of each culture was then transferred to a second set of microtiter dishes containing 10^5 macrophages per well, yielding an MOI of 5 to 10. After 3 days, the incubation medium was replaced with 0.1 ml of 10% Alamar Blue in RPMI (vol/vol); then macrophage viability was quantified as described above for the cytotoxicity assay.

To test whether the enhanced intracellular growth phenotype was linked to the *L. pneumophila* genomic clone or to the *L. micdadei* chromosome, plasmid DNA was isolated from each *L. micdadei* candidate by a standard nonalkaline procedure, followed by sequential precipitation with hexadecyltrimethylammonium bromide and ethanol (24). Plasmids that contained genomic fragments, as judged by restriction endonuclease mapping, were reintroduced into D-2676 by conjugation, then retested for the ability to confer increased growth in macrophages.

RESULTS

Intracellular replication of *Legionella*. A hallmark of *Legionella* pathogenesis is the capacity to survive and to replicate within macrophages (48, 49, 57). Therefore, entry and survival in primary bone marrow macrophages by *L. micdadei* 31B, Rivera, Camilleri, and 31B and *L. pneumophila* Lp02 were compared. Macrophages were infected for 2 h; then extracellular bacteria were killed with gentamicin and cell-associated CFU were enumerated. As observed previously (13), approximately 8% of the inoculum of post-exponential-phase *L. pneumophila* cells initiated an infection of macrophages (Fig. 1). In comparison, only 1 to 3% of the *L. micdadei* strains did so, indicating less-efficient binding, entry, and/or survival in macrophages.

Next, the ability of intracellular bacteria to replicate in mouse macrophages and in the human monocyte cell line U937 was assessed. Consistent with previous reports (57, 60), *L. pneumophila* and *L. micdadei* 31B, Rivera, and Camilleri all showed robust growth in U937 cells, with a 100-fold increase in the yield of CFU over a 2-day period (Fig. 2A). Strain D-2676

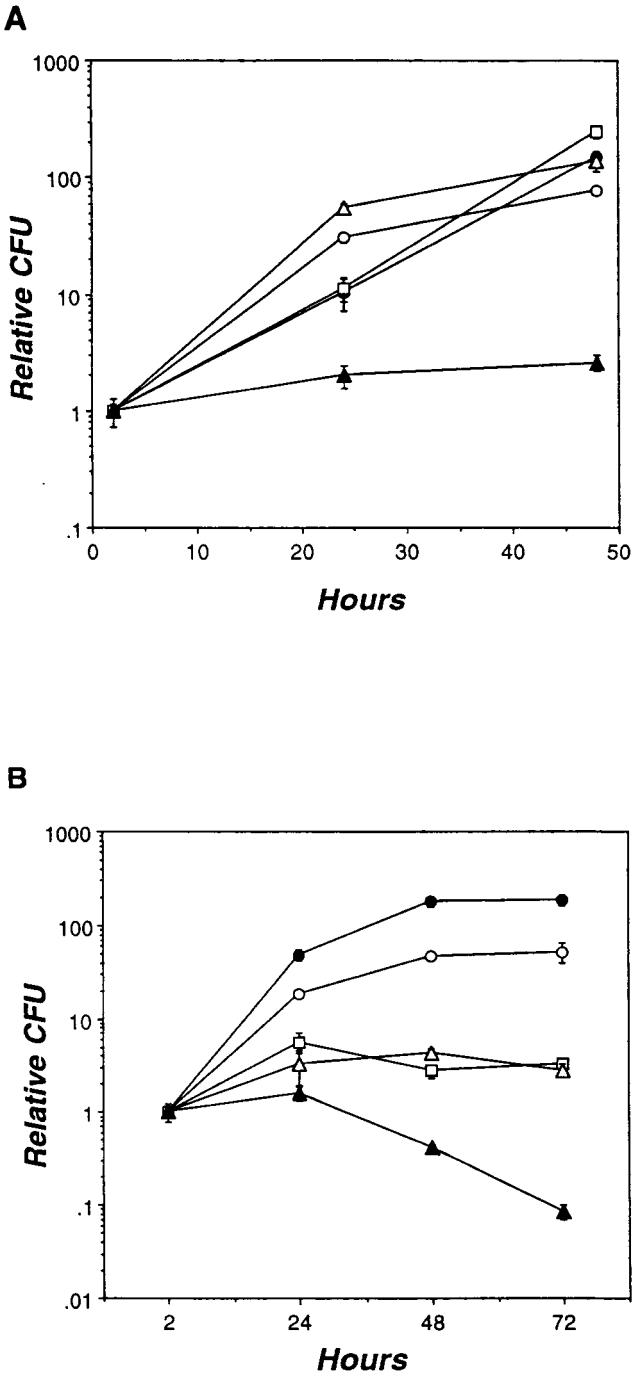

FIG. 2. Intracellular growth of *Legionella* strains. (A) U937 cells were infected with the *Legionella* strains shown; then, at the times indicated, the numbers of viable bacteria were determined. The relative number of CFU was calculated by dividing the CFU at each time point by the CFU at the first time point; the actual initial CFU was 3.5×10^4 for Lp02 (open circles), 1.4×10^4 for 31B (solid circles), 2.7×10^4 for Rivera (open squares), 1.0×10^4 for Camilleri (open triangles), and 1.1×10^4 for D-2676 (solid triangles). The mean CFU was determined from triplicate samples; the standard error of the mean is indicated by error bars. Similar results were obtained in two other experiments. (B) Macrophages were infected with the *Legionella* strains shown; then intracellular growth was determined as described above. The actual initial CFU was 2.9×10^4 for Lp02 (open circles), 8.6×10^3 for 31B (solid circles), 2.1×10^4 for Rivera (open squares), 8.5×10^3 for Camilleri (open triangles), and 2.4×10^4 for D-2676 (solid triangles). Similar results were obtained in six other experiments.

TABLE 1. Microscopic assay of intracellular growth[a]

Strain	% Infected macrophages containing the following no. of bacteria/cell:		
	1–10	11–20	>20
Lp02	50 ± 6.4	14 ± 2.0	36 ± 7.4
31B	46 ± 1.3	20 ± 3.9	34 ± 4.9
Rivera	87 ± 2.9	6.3 ± 2.2	6.7 ± 1.8
Camilleri	88 ± 2.0	4.3 ± 0.3	7.7 ± 1.9
D-2676	89 ± 4.1	4.0 ± 1.8	7.0 ± 2.3

[a] Macrophage monolayers were infected with *Legionella* strains, incubated for 18 h, fixed, and stained with the DNA dye DAPI; then the number of bacteria per macrophage was counted. Each value represents the mean fraction of at least 50 infected macrophages which contained the indicated number of bacteria. Shown are the means and the standard errors of the means determined in three experiments.

was markedly less virulent: its CFU increased only slightly. Primary mouse macrophages were generally more restrictive for *Legionella* growth (Fig. 2B). The yield of CFU for *L. pneumophila* and *L. micdadei* 31B increased approximately 100-fold during a 72-h infection. In contrast, the numbers of Rivera and Camilleri CFU increased less than 10-fold during the first 24 h, then declined slightly. Again, the least virulent strain was D-2676: 24 h after infection, its yield of CFU declined markedly. Thus, as judged by growth in primary macrophages, *L. micdadei* 31B appeared as virulent as *L. pneumophila*, D-2676 appeared attenuated, and Rivera and Camilleri had an intermediate phenotype.

Intracellular growth by individual bacteria. The lower yield of CFU in primary macrophages observed for *L. micdadei* Rivera, Camilleri, and D-2676 could indicate that the majority of cells in the inoculum may replicate at a lower rate than *L. pneumophila*. Alternatively, a subset of cells in the inoculum may replicate efficiently while the majority do not multiply at all or are killed. To differentiate between these two possibilities, intracellular replication by individual cells was analyzed microscopically under conditions which ensured that only one bacterium entered each macrophage. Late in the primary infection period, approximately one-third of the macrophages infected with *L. pneumophila* contained more bacteria than could be counted, an indication of robust intracellular growth (Table 1). As expected, strain 31B showed a similar profile, confirming its efficient replication in macrophages (Table 1; Fig. 2). In contrast, more than 85% of the macrophages infected with Rivera, Camilleri, or D-2676 contained no more than 10 bacteria, indicating that most intracellular bacteria had not replicated efficiently. Interestingly, when infected with Rivera, Camilleri, or D-2676, a small minority of macrophages (<10%) contained bacteria too numerous to count. That a subpopulation of each of these strains established a primary infection in macrophages may reflect heterogeneity in the virulence of the bacterial population; alternatively, macrophages may not respond uniformly to infection.

Three of the *L. micdadei* strains grew poorly in primary macrophages, whereas strain 31B appeared as virulent as *L. pneumophila* Lp02. To investigate whether the relative intracellular growth levels of the four *L. micdadei* strains correlated to expression levels of particular virulence traits, a detailed phenotypic comparison was performed.

Evasion of phagosome-lysosome fusion. To determine whether the reduced initiation of infection (Fig. 1) reflected decreased survival of intracellular *L. micdadei*, the interaction between macrophage lysosomes and phagosomes harboring each of the strains was examined. First, macrophage lysosomes were labeled by endocytosis of the soluble fluorescent marker TRov; 2 h after infection with post-exponential-phase *Legionella*, colocalization of bacteria and TRov was evaluated by fluorescence microscopy (77). As expected, fewer than 4% of phagosomes harboring virulent *L. pneumophila* contained the lysosomal marker (Fig. 3A). In contrast, approximately 80% of

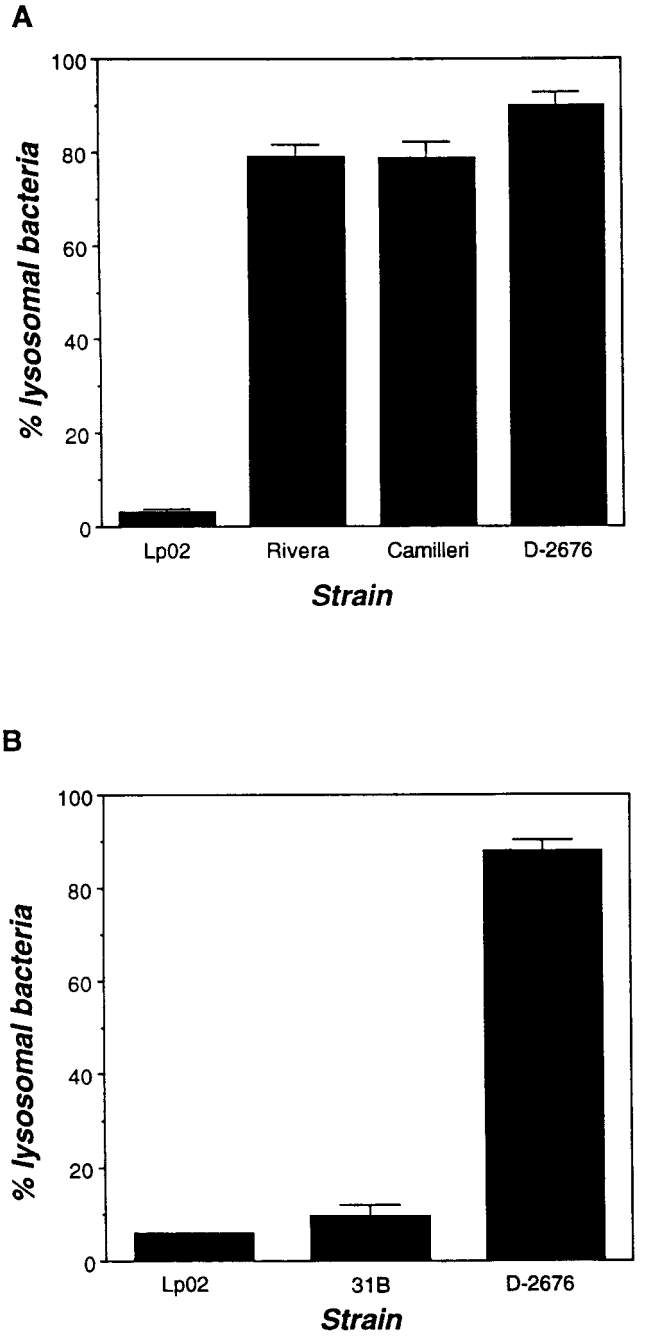

FIG. 3. Evasion of phagosome-lysosome fusion by *Legionella* strains. (A) Macrophages prelabeled with the endocytic probe TRov were infected with the *Legionella* strain indicated for 2 h; then the percentage of intracellular bacteria that colocalized with TRov was determined by fluorescence microscopy. Intracellular and extracellular bacteria were stained differentially by using *Legionella*-specific antisera (Materials and Methods). (B) Evasion of phagosome-lysosome fusion was determined essentially as described above, except that macrophages were infected with Lp02, 31B, and D-2676 which had been prelabled with FLUOS. At least 50 intracellular bacteria were scored in each experiment; shown are the means and the standard errors of the means calculated from three experiments.

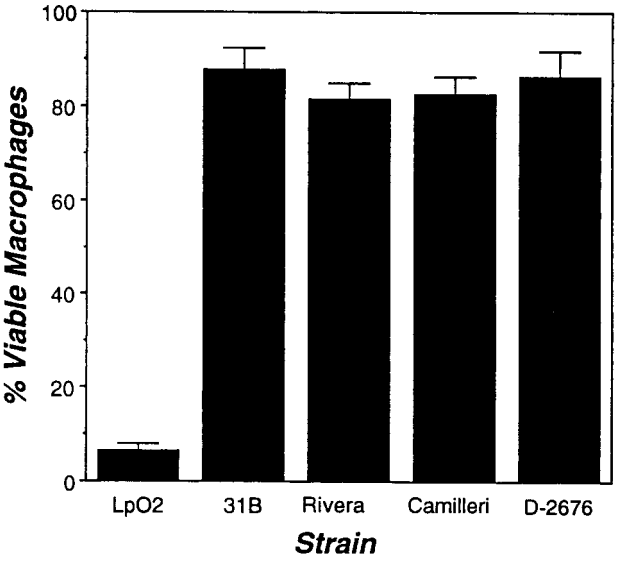

FIG. 4. Cytotoxicity of *Legionella* strains for macrophages. Macrophages were incubated for 1 h with the bacterial strain indicated; then macrophage viability was assessed by using the redox-sensitive dye Alamar Blue (see Materials and Methods). Shown are the percentages (means and standard errors) of viable macrophages infected at an MOI of 5 with Camilleri, Rivera, and 31B (n = 6), D-2676 (n = 4), and Lp02 (n = 2).

Rivera, Camilleri, and D-2676 phagosomes had fused with lysosomes (Fig. 3A), a result consistent with the lower numbers of viable intracellular bacteria 2 h after infection (Fig. 1).

Strain 31B stained poorly with the *L. micdadei*-specific antiserum, making intracellular bacteria difficult to locate by this method. Instead, strain 31B was labeled directly with the fluorescent probe FLUOS prior to infection of macrophages. As expected, only 6% of phagosomes containing FLUOS-*L. pneumophila* fused with lysosomes, but nearly 90% of FLUOS–D-2676 phagosomes colocalized with TRov (compare Fig. 3A and B). Consistent with its efficient growth in macrophages (Fig. 2A and Table 1), phagosomes harboring 31B rarely fused with lysosomes (Fig. 3B). Thus, unlike the three other *L. micdadei* strains examined, the majority of intracellular 31B bacteria escaped lysosomal killing and established a replication niche in mouse macrophages.

Cytotoxicity for macrophages. *L. pneumophila* can kill macrophages by a replication-independent but contact-dependent mechanism (41). Cytotoxicity has been correlated with *L. pneumophila* virulence, although its role in pathogenesis is not known (46). To measure *L. micdadei* cytotoxicity, macrophages and bacteria were cocultured for 1 h; then macrophage viability was measured by using the colorimetric dye Alamar Blue (51). As expected, at an MOI greater than 5, *L. pneumophila* killed approximately 95% of the macrophage monolayer (Fig. 4). In contrast, none of the *L. micdadei* strains were cytotoxic (Fig. 4). Even at the high MOI of 50, neither exponential- nor post-exponential-phase cultures of *L. micdadei* affected macrophage viability (data not shown). Surprisingly, even strain 31B, which replicates efficiently in macrophages, lacked contact-dependent cytotoxicity. Therefore, if the cytotoxicity of *L. pneumophila* is critical for establishment of its unique replication niche (45) and/or escape from amino acid-depleted host cells (13), *L. micdadei* must use an alternative strategy.

Sodium sensitivity. The ability of *L. pneumophila* to survive and to replicate in macrophages has been correlated with Na^s (15, 69, 82), a trait expressed in the post-exponential phase (13). Yet two broad surveys of *Legionella* indicate that this correlation is not complete (2, 58). To assess the association between the virulence and Na^s of *L. micdadei*, bacteria collected from exponential- and post-exponential-phase cultures were plated onto CYE which did or did not contain 100 mM NaCl; then colony formation was quantified. Consistent with the differences observed between virulent and avirulent *L. pneumophila*, the severely attenuated *L. micdadei* strain D-2676 was Na^r, while the most virulent strain, 31B, was relatively Na^s in both the exponential and the post-exponential phase (Fig. 5). Rivera and Camilleri, which appear to be partially attenuated, were slightly Na^s during the exponential phase and more so in the post-exponential period, consistent with a previous report (58). In general, two rules held: relative Na^s correlated with virulence, and the Na^s of each of the strains increased in the post-exponential phase.

Conjugal transfer of DNA. It has been proposed that *L. pneumophila* secretes virulence factors via the putative Dot-Icm complex, which also mediates the conjugal transfer of plasmid DNA (73, 80). To determine whether conjugation activity correlates with the virulence of *L. micdadei*, we compared the conjugation efficiencies of the four strains of *L. micdadei* with that of *L. pneumophila*. As predicted by the Dot-Icm secretion model (73, 80), the virulent *L. micdadei* strain 31B transferred DNA to the recipient more efficiently than *L. pneumophila*, whereas the three attenuated *L. micdadei* strains donated DNA at a rate approximately 10-fold lower than that of *L. pneumophila* (Fig. 6). In contrast, *L. pneumophila dotB* mutants donate plasmid DNA 100- to 1,000-fold less efficiently than the wild type (80) (data not shown). Whether transfer of plasmid DNA by *L. micdadei* is mediated by a Dot-Icm complex remains to be tested.

Osmotic resistance. Like *E. coli* (43), *L. pneumophila* becomes osmotically resistant as it exits the exponential phase (13). A similar phenotypic switch was observed for all four strains of *L. micdadei*. As cultures of each of the strains entered the post-exponential phase, their osmotic resistance in-

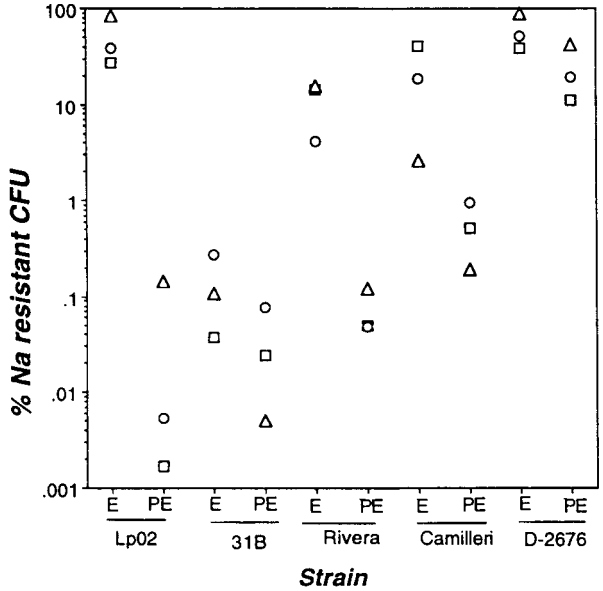

FIG. 5. Sodium sensitivities of *Legionella* strains. Bacteria cultured to the exponential (E) and post-exponential (PE) phase were plated in duplicate onto CYE or CYET to quantify total CFU and onto CYE or CYET containing 100 mM NaCl to enumerate sodium-resistant CFU. Shown is the percent plating efficiency on sodium-containing medium determined in three experiments, each represented by a unique symbol (open circles, open squares, or solid triangles).

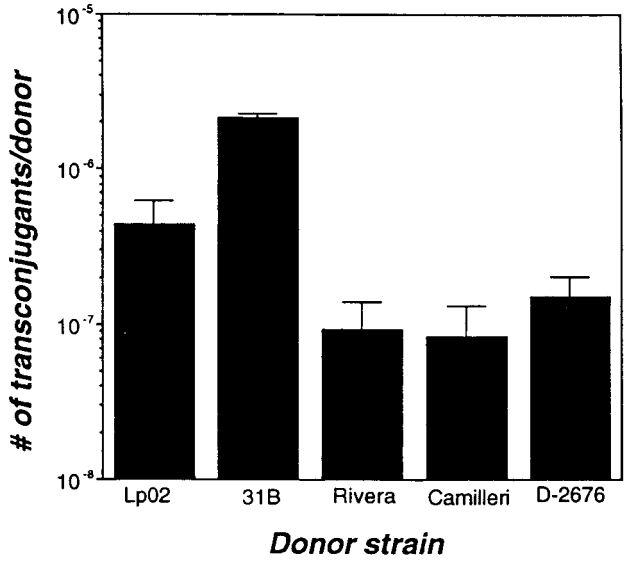

FIG. 6. Conjugal DNA transfer. *Legionella* organisms cultured to exponential phase were mixed with an approximately equal number of CFU of the recipient strain DH5α, then incubated for 4 h. Appropriate dilutions of the mating mix were plated in duplicate onto LB-kanamycin plates to quantify conjugation efficiency. Shown is the number (mean and standard error) of conjugants obtained per donor in one experiment; similar results were obtained in at least one other experiment. The difference in conjugal efficiency between *L. pneumophila* and the *L. micdadei* strains was significant ($P < 0.001$ by the χ^2 test of independence).

creased approximately 10-fold (Fig. 7). Thus, osmotic resistance correlated with the growth phase, but not the virulence, of *L. micdadei*.

Gain-of-function genetic screen for *L. pneumophila* virulence factors. Two observations indicated that the *L. micdadei* strains are equipped for intracellular growth: all of the strains

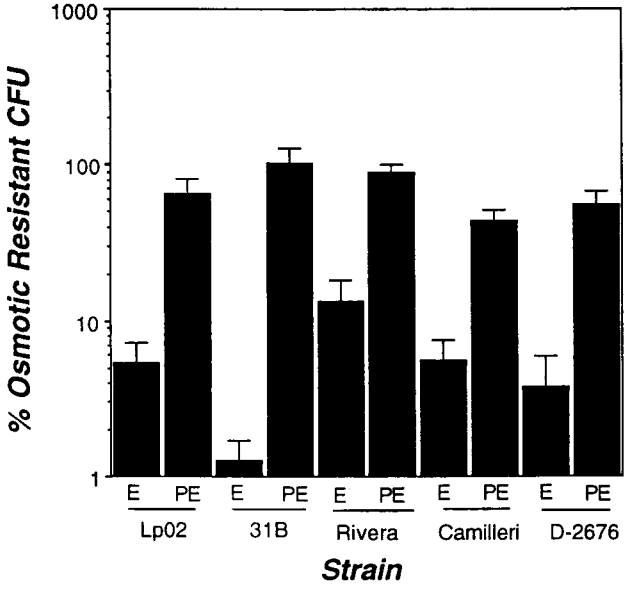

FIG. 7. Osmotic sensitivity of *Legionella*. Exponential- and post-exponential-phase cultures of *Legionella* were incubated for 1 h in broth with or without 0.3 M KCl, diluted into distilled water, then plated in duplicate onto CYE or CYET to quantify CFU. Shown are the means and standard errors of the means obtained in six experiments.

TABLE 2. Genetic screen for *L. pneumophila* loci which stimulate *L. micdadei* intracellular growth

Pool (no. of members)[a]	Enrichment cycle 1			Enrichment cycle 2		
	CFU at 0 h	CFU at 72 h	% Recovery[b]	CFU at 0 h	CFU at 72 h	% Recovery[b]
A (1.5×10^4)	5.1×10^6	6.0×10^3	0.12	6.4×10^6	2.9×10^3	0.05
B (1.8×10^4)	2.2×10^6	6.0×10^3	0.29	3.7×10^6	5.0×10^3	0.14
C (1.6×10^4)	3.3×10^6	7.5×10^3	0.23	11×10^6	5.2×10^3	0.05

[a] Based on an average insert size of 5 to 10 kb and an estimated *L. pneumophila* genome size of 4×10^6, each pool represents the equivalent of 16 to 19 complete genomes.

[b] Based on measures of macrophage infection (Fig. 1) and replication (Fig. 2A), the predicted percent recovery for the parent strain, D-2676, is 0.5%.

replicated in the U937 cell line (Fig. 2A), and a subpopulation of each exhibited robust growth in primary macrophages (Table 1). Yet, in nearly every phenotypic test, *L. micdadei* appeared less virulent than *L. pneumophila*. In an attempt to identify the genetic basis for this difference, we designed a gain-of-function, interspecies complementation strategy applied previously for a variety of pathogenesis model systems (4, 20, 23, 42, 54, 59, 75, 85). In particular, we screened an *L. pneumophila* genomic library for loci which conferred a robust intracellular growth phenotype on *L. micdadei*. To maximize the selective pressure exerted by the macrophages, we chose as the parent strain D-2676, a clinical isolate which is killed in macrophage lysosomes (Fig. 2B and 3).

To enrich for genomic clones which conferred increased intracellular survival and/or replication on *L. micdadei*, pools of D-2676 transconjugants were passaged twice in macrophage cultures. On the basis of previous infection and intracellular growth assays (Fig. 1 and 2A), we estimated that approximately 0.5% of the parental strain would survive each enrichment cycle. Judging from the percent recovery of each enrichment, none of the transconjugant pools appeared to contain strains with increased virulence (Table 2).

To examine the pools obtained after enrichment in more detail, a total of 405 strains collected from each of the enrichment pools were screened individually for their ability to kill macrophages. Eighteen candidates exhibited a modest increase in macrophage killing, as measured by the Alamar Blue viability technique (see Materials and Methods). To determine whether the increased cytotoxicity was likely conferred by an *L. pneumophila* or an *L. micdadei* locus, the plasmid-borne *L. pneumophila* genomic clones were analyzed by restriction endonuclease mapping. Surprisingly, 13 of 18 candidate plasmids had sustained an extensive deletion and/or lacked an *L. pneumophila* insert. Moreover, upon retransformation of the parent, D-2676, none of the remaining five plasmids, which contained genomic fragments of varying sizes, conferred increased killing of macrophages. Therefore, passage in macrophage culture appeared to enrich for two classes of mutations: *L. micdadei* chromosomal mutations which enhanced virulence and plasmid mutations which relieved a growth inhibition, perhaps encoded by the original *L. pneumophila* genomic fragment or exerted by mobilization sequences of the IncQ RSF1010 plasmid (73). Neither class of mutants was analyzed further.

DISCUSSION

We took a comparative approach toward understanding the pathogenesis of the two causative agents of Legionnaire's disease, *L. pneumophila* and *L. micdadei*. Using a series of quan-

TABLE 3. Summary of *L. pneumophila* and *L. micdadei* phenotypes

Strain	Growth in U937 cells[a]	Growth in macrophages[b]	Entry and survival[c]	Evasion of lysosomes[d]	Cytotoxicity[e]	Sodium sensitivity[f]	Conjugation[g]	Osmotic resistance[h]
L. pneumophila Lp02	+++	++	++	+++	+	+	++	+
L. micdadei								
31B	+++	+++	+	+++	−	+	+++	+
Rivera	+++	+	+	+	−	+	+	+
Camilleri	+++	+	+	+	−	+	+	+
D-2676	+	−	+	+	−	−	+	+

[a] See Fig. 1A.
[b] See Fig. 1B.
[c] See Fig. 2.
[d] See Fig. 3.
[e] See Fig. 4.
[f] See Fig. 5; phenotype of post-exponential-phase cells.
[g] See Fig. 6; phenotype of post-exponential-phase cells.
[h] See Fig. 7; phenotype of post-exponential-phase cells.

titative phenotypic assays, we sought to identify those traits most likely to be required by *Legionella* for robust growth in macrophages. In nearly every test, *L. micdadei* strains appeared less virulent than *L. pneumophila* (Table 3). One hypothesis consistent with these results is that *L. pneumophila* and *L. micdadei* encode the same battery of virulence factors, but *L. micdadei* lacks a factor critical for high-level expression of one or more traits. Attempts to augment the intracellular growth of *L. micdadei* by providing *L. pneumophila* genomic fragments in trans were not successful (Table 2). Therefore, simplistic models in which a single virulence determinant or locus differentiates these pathogenic *Legionella* spp. were not supported. Instead, results of this study and others (62, 83) suggest that *L. pneumophila* and *L. micdadei*, though similar in some respects, use different strategies to parasitize host cells.

L. pneumophila expresses a contact-dependent cytotoxicity (41) concomitant with exit from the exponential phase of growth (13). Recently, a pore-forming activity that correlated with *L. pneumophila* virulence was characterized. When added to either erythrocytes or macrophages at a high MOI, *L. pneumophila* organisms insert a pore which leads to osmotic lysis and rapid cell death (46). Under normal infection conditions, *L. pneumophila* may insert pores into its nascent phagosomal membrane to block, by some undefined mechanism, subsequent fusion with the lysosomal network (45). Alternatively, or additionally, the cytotoxin may facilitate the lysis of host membranes by starved bacteria, which must escape to locate a new supply of nutrients (13). Surprisingly, none of the *L. micdadei* strains were cytotoxic for macrophages (Fig. 4), yet three of the strains replicated efficiently in U937 cells, and strain 31B also exhibited robust growth in the more-stringent environment of primary mouse macrophage cultures (Fig. 2). Moreover, strain 31B efficiently evaded phagosome-lysosome fusion in macrophages (Fig. 3B). Therefore, *L. micdadei* and *L. pneumophila* may establish and/or escape from replication vacuoles by different mechanisms. Alternatively, cytotoxicity may be dispensable for *L. pneumophila* growth in macrophages, despite a genetic or regulatory linkage to virulence (45, 46).

The *L. pneumophila dot* and *icm* genes are required for intracellular growth and for efficient conjugal transfer of plasmid DNA (71, 81). Thus, like *A. tumefaciens* (17) and *B. pertussis* (84), *L. pneumophila* may use a type IV secretion system to export virulence factors. A functional Dot-Icm apparatus has been correlated genetically with two other *L. pneumophila* virulence traits: Na^s and contact-dependent cytotoxicity (69, 81, 82). By one model, export of a cytotoxin or other virulence factors by the Dot-Icm transport apparatus permits the accumulation of inhibitory levels of NaCl in the cytosol; mutational inactivation of this machinery confers salt resistance, loss of cytotoxicity, and avirulence (82). In our studies of *L. micdadei*, a strict correlation between conjugation efficiency, Na^s, and cytotoxicity was not observed. For example, although the Rivera, Camilleri, and D-2676 strains of *L. micdadei* appeared similarly competent to transfer plasmid DNA by conjugation (Fig. 6), their degrees of Na^s differed (Fig. 5), and none were cytotoxic (Fig. 4). Apparently, conjugal transport systems of *Legionella* do not necessarily confer Na^s.

To begin to address whether *L. micdadei* encodes a Dot-Icm transport system, we tested whether *L. micdadei* contained genomic sequences homologous to *dotA*, *dotB*, *dotE*, *dotF*, or *dotG* of *L. pneumophila*. Under low-stringency Southern hybridization conditions which allowed a 30% base pair mismatch (T_m − 47°C) (3), full-length probes of *dotA*, *dotB*, *dotE*, and *dotFG* genes each hybridized with genomic fragments from each of the four *L. micdadei* strains (44). When the stringency was increased to allow a 20% base pair mismatch (T_m − 30°C) (3), only the *dotA* probe hybridized with *L. micdadei* genomic DNA. These data indicate that *L. micdadei* harbors sequences homologous to both *dot-icm* region I and region II (71). Whether *L. micdadei* encodes functional copies of the 23 *dot-icm* genes or assembles a functional Dot-Icm complex important for conjugation or intracellular growth remains to be established.

In macrophages and amoebae, *L. pneumophila* replicates in a compartment bounded by the ER (33, 37, 77). Based on morphological and kinetic studies of replication vacuole formation, Swanson and Isberg had proposed that *L. pneumophila* acquires a rich supply of nutrients by stimulating the eukaryotic autophagy pathway (77). In contrast, none of the *L. micdadei* strains examined to date appear to associate with the ER (83); instead, *L. micdadei* occupies a dilated phagosome (62). According to the autophagy exploitation model, the inability of *L. micdadei* to associate with the ER to obtain nutrients could account for its poor intracellular growth. However, *L. micdadei* 31B, which replicated in macrophages as efficiently as did *L. pneumophila*, occupied a dilated phagosome which was not associated with the ER, as judged by immunofluorescence microscopic localization of the ER luminal protein Bip (44, 77). Therefore, even though ER association has been correlated with the intracellular growth of *L. pneumophila* (37, 77, 79), it does not appear to play a role in *L. micdadei* pathogenesis. Thus, the intriguing question of how intracellular *Legionella*

organisms obtain the amino acids needed for replication remains.

Designation of 31B as *L. micdadei* was suspect because its intracellular growth characteristics differed from those of the three other *L. micdadei* strains studied and because it stained poorly with the *L. micdadei*-specific antiserum. In particular, immunofluorescent staining of broth-grown 31B required four-fold-higher antiserum concentrations to achieve the intensity seen for Rivera, Camilleri, and D-2676. Interestingly, genetic divergence of 31B was also suggested by Southern hybridization analysis, which revealed restriction fragment polymorphisms in the chromosomal loci homologous to *L. pneumophila dotA*, *dotB*, *dotE*, and *dotFG* for 31B compared to the other three *L. micdadei* strains. On the other hand, 31B did not react strongly with *L. pneumophila*-specific antiserum (data not shown). Furthermore, previous molecular studies of the Mip gene and protein of strain 31B established its relatedness to several other *L. micdadei* clinical isolates (57). Additional molecular genetic studies are required to explain the potent virulence of 31B relative to other clinical isolates of *L. micdadei*.

Taken together, our data indicate that the differential virulence of *L. pneumophila* and *L. micdadei* is not attributable to a single phenotypic or genetic trait. It remains possible that *L. micdadei* lacks a particular genetic determinant which is critical for robust intracellular growth but which either was not represented in the *L. pneumophila* library, was poorly expressed by *L. micdadei*, or was not sufficient to bypass the more extensive genetic differences of D-2676. However, we favor the alternative view that *Legionella* is a diverse genus whose species appear to use quite different strategies to parasitize host cells. Perhaps the most intriguing questions raised by this comparative study are whether cytotoxicity, sodium sensitivity, the conjugation apparatus, and the host ER contribute to *L. pneumophila* pathogenesis, and if so, how.

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REFERENCES

1. **Andrews, H. L., J. P. Vogel, and R. R. Isberg.** 1998. Identification of linked *Legionella pneumophila* genes essential for growth and evasion of the endocytic pathway. Infect. Immun. **66:**950–958.
2. **Arakawa, M., Y. Hashimoto, T. Ezaki, and E. Yabuuchi.** 1992. Virulence of *Legionella* species does not correlate with their sodium chloride resistance. Med. Microbiol. Lett. **1:**30–37.
3. **Ausubel, F. M., R. B. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
4. **Banerjee, A., E. Dubnau, A. Quermard, V. Balasubramanian, K. S. Um, T. Wilson, D. Collins, G. de Lisle, and W. R. Jacobs.** 1994. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science **263:**227–230.
5. **Bangsborg, J. M., M. T. Collins, N. Hoiby, and P. Hindersson.** 1989. Cloning and expression of the *Legionella micdadei* "common antigen" in *Escherichia coli*. APMIS **97:**14–22.
6. **Bangsborg, J. M., P. Hindersson, G. Shand, and N. Hoiby.** 1995. The *Legionella micdadei* flagellin gene: expression in *Escherichia coli* K12 and DNA sequence of the gene. APMIS **103:**869–877.
7. **Benson, R. F., and B. S. Fields.** 1998. Classification of the genus *Legionella*. Semin. Respir. Infect. **13:**90–99.
8. **Berger, K. H., and R. R. Isberg.** 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol. Microbiol. **7:**7–19.
9. **Berger, K. H., J. J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dotA* gene. Mol. Microbiol. **14:**809–822.
10. **Brenner, D.** 1987. Classification of legionellae. Semin. Respir. Infect. **2:**190–205.
11. **Brown, M. R. W.** 1999. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. Trends Microbiol. **7:**46–50.
12. **Byrd, T. F., and M. A. Horwitz.** 1988. Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. J. Clin. Investig. **83:**1457–1465.
13. **Byrne, B., and M. S. Swanson.** 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. Infect. Immun. **66:**3029–3034.
14. **Carratala, J., F. Gudiol, R. Pallares, J. Dorca, R. Verdaguer, J. Ariza, and F. Manresa.** 1994. Risk factors for nosocomial *Legionella pneumophila* pneumonia. Am. J. Respir. Crit. Care Med. **149:**625–629.
15. **Catrenich, C. E., and W. Johnson.** 1989. Characterization of the selective inhibition of growth of virulent *Legionella pneumophila* by supplemented Mueller-Hinton medium. Infect. Immun. **57:**1862–1864.
16. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type-I specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. USA **93:**14648–14653.
17. **Christie, P. J.** 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in *Eubacteria*. J. Bacteriol. **179:**3085–3094.
18. **Cianciotto, N. P., and B. S. Fields.** 1992. *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. Proc. Natl. Acad. Sci. USA **89:**5188–5191.
19. **Cianciotto, N. P., R. Long, B. I. Eisenstein, and N. C. Engleberg.** 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates the initiation of intracellular infection. Infect. Immun. **57:**1255–1262.
20. **Clark-Curtiss, J. E.** 1998. Identification of virulence determinants in pathogenic mycobacteria. Curr. Top. Microbiol. Immunol. **225:**57–79.
21. **Clemens, D. L., and M. A. Horwitz.** 1993. Hypoexpression of major histocompatibility complex molecules on *Legionella pneumophila* phagosomes and phagolysosomes. Infect. Immun. **61:**2803–2812.
22. **Clemens, D. L., and M. A. Horwitz.** 1992. Membrane sorting during phagocytosis: selective exclusion of major histocompatibility complex molecules but not complement receptor CR3 during conventional and coiling phagocytosis. J. Exp. Med. **175:**1317–1326.
23. **Collins, D. M., R. P. Kawakami, G. W. de Lisle, L. Pascopella, B. R. Bloom, and W. R. Jacobs.** 1995. Mutation of the principal σ factor causes loss of virulence in a strain of the *Mycobacterium tuberculosis* complex. Proc. Natl. Acad. Sci. USA **92:**8036–8040.
24. **Del Sal, G., G. Manfioletti, and C. Schneider.** 1989. The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages, or plasmids suitable for sequencing. BioTechniques **7:**514–519.
25. **Donowitz, G. R., I. Reardon, J. Dowling, L. Rubin, and D. Focht.** 1990. Ingestion of *Legionella micdadei* inhibits human neutrophil function. Infect. Immun. **58:**3307–3311.
26. **Dowling, J. N., A. K. Saha, and R. H. Glew.** 1992. Virulence factors of the family *Legionellaceae*. Microbiol. Rev. **56:**32–60.
27. **Fang, G., V. L. Yu, and R. M. Vickers.** 1989. Disease due to the *Legionellaceae* (other than *Legionella pneumophila*). Medicine **68:**116–132.
28. **Fields, B. S.** 1996. The molecular ecology of legionellae. Trends Microbiol. **4:**286–290.
29. **Fields, B. S., J. M. Barbaree, E. B. Shotts, Jr., J. C. Feeley, W. E. Morrill, G. N. Sanden, and M. J. Dykstra.** 1986. Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. Infect. Immun. **53:**553–559.
30. **Fox, K. F., and A. Brown.** 1993. Properties of the genus *Tatlockia*. Differentiation of *Tatlockia* (*Legionella*) *maceachernii* and *micdadei* from each other and from other legionellae. Can. J. Microbiol. **39:**486–491.
31. **Fraser, D. W., T. R. Tsai, W. Orenstin, W. E. Parken, H. J. Beechan, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, and P. S. Brachman.** 1977. Legionnaires' disease: description of an epidemic of pneumonia. N. Engl. J. Med. **297:**1189–1197.
32. **Fry, N. K., S. Warwick, N. A. Saunders, and M. T. Embley.** 1991. The use of 16S ribosomal RNA analysis to investigate the phylogeny of the family *Legionellaceae*. J. Gen. Microbiol. **137:**1215–1222.
33. **Gao, L. Y., O. S. Harb, and Y. Abu Kwaik.** 1997. Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infect. Immun. **65:**4738–4746.
34. **Haley, C. E., M. L. Cohen, J. Halter, and R. D. Meyer.** 1979. Nosocomial Legionnaire's disease: a continuing common-source epidemic at the Wadsworth Medical Center. Ann. Intern. Med. **90:**583–586.
35. **Heuner, K., L. Bender-Beck, B. C. Brand, P. C. Luck, K.-H. Mann, R. Marre, M. Ott, and J. Hacker.** 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. Infect. Immun. **63:**2499–2507.
36. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Investig. **66:**441–450.

37. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
38. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
39. **Horwitz, M. A.** 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36:**27–33.
40. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99:**1936–1943.
41. **Husmann, L. K., and W. Johnson.** 1994. Cytotoxicity of extracellular *Legionella pneumophila*. Infect. Immun. **62:**2111–2114.
42. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K12. Nature **317:**262–264.
43. **Jenkins, D. E., S. A. Chaisson, and A. Matin.** 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. **172:** 2779–2781.
44. **Joshi, A. D., and M. S. Swanson.** Unpublished data.
45. **Kirby, J. E., and R. R. Isberg.** 1998. Legionnaire's disease: the pore macrophage and the legion of terror within. Trends Microbiol. **6:**256–258.
46. **Kirby, J. E., J. P. Vogel, H. L. Andrews, and R. R. Isberg.** 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Mol. Microbiol. **27:**323–336.
47. **Korvick, J. A., V. L. Yu, and G.-D. Fang.** 1987. *Legionella* species as hospital-acquired respiratory pathogens. Semin. Respir. Infect. **2:**34–47.
48. **Levi, M. H., A. W. Pasculle, and J. N. Dowling.** 1987. Role of alveolar macrophage in host defense and immunity to *Legionella micdadei* pneumonia in the guinea pig. Microb. Pathog. **2:**269–282.
49. **Liles, M. R., P. H. Edelstein, and N. P. Cianciotto.** 1999. The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen *Legionella pneumophila*. Mol. Microbiol. **31:**959–970.
50. **Marston, B. J., H. B. Lipman, and R. F. Breiman.** 1994. Surveillance for Legionnaires' disease. Arch. Intern. Med. **154:**2417–2422.
51. **McClain, M. S., M. C. Hurley, J. K. Brieland, and N. C. Engleberg.** 1996. The *Legionella pneumophila hel* locus encodes intracellularly induced homologs of heavy-metal ion transporters of *Alcaligenes* spp. Infect. Immun. **64:** 1532–1540.
52. **McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, and W. R. Dowdle.** 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory diseases. N. Engl. J. Med. **297:** 1197–1203.
53. **McLean, I. W., and P. K. Nakane.** 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. J. Histochem. Cytochem. **22:**1077–1083.
54. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:** 1242–1248.
55. **Muder, R. R., V. L. Yu, and J. J. Zuravleff.** 1983. Pneumonia due to the Pittsburgh pneumonia agent: new clinical perspective with a review of the literature. Medicine **62:**120–128.
56. **Myerowitz, R. L., A. W. Pasculle, J. N. Dowling, G. J. Pazin, M. Puerzer, R. B. Yee, C. R. Rinaldo, and T. R. Hakala.** 1979. Opportunistic lung infection due to "Pittsburgh pneumonia agent". N. Engl. J. Med. **301:**953–958.
57. **O'Connell, W., J. M. Bangsborg, and N. P. Cianciotto.** 1995. Characterization of a *Legionella micdadei mip* mutant. Infect. Immun. **63:**2840–2845.
58. **O'Connell, W. A., L. Dhand, and N. P. Cianciotto.** 1996. Infection of macrophage-like cells by *Legionella* species that have not been associated with disease. Infect. Immun. **64:**4381–4384.
59. **Pascopella, L., F. M. Collins, J. M. Martin, M. H. Lee, G. F. Hatfull, C. K. Stover, B. R. Bloom, and W. R. Jacobs.** 1994. Use of in vivo complementation in *Mycobacterium tuberculosis* to identify a genomic fragment associated with virulence. Infect. Immun. **62:**1313–1319.
60. **Pearlman, E., N. C. Engleberg, and B. I. Eisenstein.** 1988. Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. Microb. Pathog. **5:**87–95.
61. **Plikaytis, B. B., G. M. Carlone, C.-P. Pau, and H. W. Wilkinson.** 1987. Purified 60-kilodalton *Legionella* protein antigen with *Legionella*-specific and nonspecific epitopes. J. Clin. Microbiol. **25:**2080–2084.
62. **Rechnitzer, C., and J. Blom.** 1989. Engulfment of the Philadelphia strain of *Legionella pneumophila* within the pseudopod coils in human phagocytes. Comparison with other *Legionella* strains and species. APMIS **97:**105–114.
63. **Reingold, A. L., B. M. Thomason, B. J. Brake, L. Thacker, H. W. Wilkinson, and J. N. Kuritsky.** 1984. *Legionella pneumophila* in the United States: the distribution of serogroups and species causing human illness. J. Infect. Dis. **149:**819.
64. **Rogers, B. H., G. R. Donowitz, G. K. Walker, S. A. Harding, and M. A. Sande.** 1979. Opportunistic pneumonia. A clinicopathological study of five cases by an unidentified acid-fast bacterium. N. Engl. J. Med. **301:**959–961.
65. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33:**1179–1183.
66. **Roy, C. R., K. H. Berger, and R. R. Isberg.** 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. **28:**663–674.
67. **Roy, C. R., and R. R. Isberg.** 1997. Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. Infect. Immun. **65:**571–578.
68. **Rudin, J. E., and E. J. Wing.** 1984. A comparative study of *Legionella micdadei* and other nosocomial acquired pneumonia. Chest **86:**675–680.
69. **Sadosky, A. B., L. A. Wiater, and H. A. Shuman.** 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect. Immun. **61:**5361–5373.
70. **Segal, G., and H. A. Shuman.** 1997. Characterization of a new region required for macrophage killing by *Legionella pneumophila*. Infect. Immun. **65:** 5057–5066.
71. **Segal, G., and H. A. Shuman.** 1998. How is the intracellular fate of the *Legionella pneumophila* phagosome determined? Trends Microbiol. **6:**253–255.
72. **Segal, G., and H. A. Shuman.** 1999. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. Infect. Immun. **67:**2117–2124.
73. **Segal, G., and H. A. Shuman.** 1998. Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components of IncQ plasmid RSF1010. Mol. Microbiol. **30:**197–208.
74. **Skerrett, S. J., and T. R. Martin.** 1996. Roles for tumor necrosis factor alpha and nitric oxide in resistance of rat alveolar macrophages to *Legionella pneumophila*. Infect. Immun. **64:**3236–3243.
75. **Snapper, S. B., R. E. Melton, M. T. Kieser, and W. R. Jacobs.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
76. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science **263:**678–681.
77. **Swanson, M. S., and R. R. Isberg.** 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect. Immun. **63:** bp3609–3620.
78. **Swanson, M. S., and R. R. Isberg.** 1996. Analysis of the intracellular fate of *Legionella pneumophila* mutants by fluorescence microscopy. Ann. N. Y. Acad. Sci. **797:**8–18.
79. **Swanson, M. S., and R. R. Isberg.** 1996. Identification of *Legionella pneumophila* mutants that have aberrant intracellular fates. Infect. Immun. **64:** 2585–2594.
80. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:** bp873–876.
81. **Vogel, J. P., and R. R. Isberg.** 1999. Cell biology of *Legionella pneumophila*. Curr. Opin. Microbiol. **2:**30–34.
82. **Vogel, J. P., C. Roy, and R. R. Isberg.** 1996. Use of salt to isolate *Legionella pneumophila* mutants unable to replicate in macrophages. Ann. N. Y. Acad. Sci. **797:**271–272.
83. **Weinbaum, D. L., R. R. Benner, J. N. Dowling, A. Alpern, A. W. Pasculle, and G. R. Donowitz.** 1984. Interaction of *Legionella micdadei* with human monocytes. Infect. Immun. **46:**68–73.
84. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA **90:**2970–2974.
85. **Wilson, T. M., G. W. de Lisle, and D. M. Collins.** 1995. Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium avium*. Mol. Microbiol. **15:**1009–1015.

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