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Identification of *Legionella pneumophila*-Specific Genes by Genomic Subtractive Hybridization with *Legionella micdadei* and Identification of *lpnE*, a Gene Required for Efficient Host Cell Entry†

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Legionella pneumophila **is a ubiquitous environmental organism and a facultative intracellular pathogen of humans. To identify genes that may contribute to the virulence of *L. pneumophila*, we performed genomic subtractive hybridization between *L. pneumophila* serogroup 1 strain 02/41 and *L. micdadei* strain 02/42. A total of 144 *L. pneumophila*-specific clones were sequenced, revealing 151 genes that were absent in *L. micdadei* strain 02/42. Low-stringency Southern hybridization was used to determine the distribution of 41 sequences, representing 40 open reading frames (ORFs) with a range of putative functions among *L. pneumophila* isolates of various serogroups as well as strains of *Legionella longbeachae*, *L. micdadei*, *Legionella gormanii*, and *Legionella jordanis*. Twelve predicted ORFs were *L. pneumophila* specific, including the gene encoding the *dot*/*icm* effector, *lepB*, as well as several genes predicted to play a role in lipopolysaccharide biosynthesis and cell wall synthesis and several sequences with similarity to virulence-associated determinants. A further nine predicted ORFs were in all *L. pneumophila* serotypes tested and an isolate of *L. gormanii*. These included *icmD*, the 5′ end of a *pilMNOPQ* locus, and two genes known to be upregulated during growth within macrophages, *cadA2* and *ceaA*. Disruption of an *L. pneumophila*-specific gene (lpg2222 locus tag) encoding a putative protein with eight tetratricopeptide repeats resulted in reduced entry into the macrophage-like cell line, THP-1, and the type II alveolar epithelial cell line, A549. The gene was subsequently renamed *lpnE*, for "*L. pneumophila* entry." In summary, this investigation has revealed important genetic differences between *L. pneumophila* and other *Legionella* species that may contribute to the phenotypic and clinical differences observed within this genus.**

Legionella pneumophila is the major causative agent of Legionnaires' disease, a severe form of acute pneumonia that is responsible for 2 to 15% of cases of community-acquired pneumonia requiring hospitalization (57). *L. pneumophila* ubiquitously inhabits soil, biofilm, and aquatic environments, where it replicates within protozoa (25, 40, 49, 52, 55). Following the inhalation of aerosols containing *L. pneumophila*, the bacteria are internalized by alveolar macrophages and epithelial cells, where they replicate within an intracellular vacuole. The *Legionella* vacuole recruits the host GTPase, Rab1, and the v-SNARE, Sec22b, to establish a replicative vacuole surrounded by rough endoplasmic reticulum (RER) (30). The recruitment of RER correlates strongly with the virulence of *L. pneumophila*, and the manipulation of host cell trafficking pathways allows the bacteria to replicate to high numbers inside cells before eventual bacterial egress, host cell death, and the infection of new host cells (2, 39).

The establishment of the unique *Legionella*-containing vacuole (LCV) depends on the *dot* ("defective in organelle trafficking")/*icm* ("intracellular multiplication") type IV secretion system (8, 53), which secretes and translocates bacterial effector proteins to the LCV membrane and presumably to the cytosol of the host cell (44). Recently, several *dot*/*icm*-translocated effector proteins were identified, including a guanine nucleotide exchange factor, RalF, that recruits the host GTPase Arf1 to the LCV membrane; a large coiled-coiled protein of unknown function termed LidA; two effectors, LepA and LepB, with low homology to host SNARE proteins; and a group of DotF binding proteins, SidA to -H (14, 17, 34, 44). Interestingly, mutation of the genes encoding these proteins does not result in a significant reduction in intracellular replication within macrophages, suggesting there is much functional redundancy among these effectors (14, 17, 34, 44).

While there are more than 40 named species of *Legionella*, 80 to 90% of cases of Legionnaires' disease are caused by *L. pneumophila* serogroup 1 (7, 35, 59). *Legionella micdadei* and *Legionella longbeachae* are the next most common etiological agents of Legionnaires' disease and together account for approximately 2 to 5% of the disease worldwide (6, 43, 59). Interestingly, this trend does not hold true in Australia and New Zealand, where approximately 30% of Legionnaires' disease is attributed to *L. longbeachae* (59). Nevertheless, these epidemiological differences suggest that *L. pneumophila*, in particular serogroup 1, is more virulent than other *Legionella* species.

There have been several studies comparing the virulence traits of different *Legionella* spp., yet little is known about the genetic basis of these phenotypic differences. Unlike *L. pneumophila*, *L. micdadei* appears to replicate within a vacuole that

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does not recruit host RER and undergoes phagosome-lysosome fusion (26, 29, 48). Both *L. longbeachae* and *L. micdadei* replicate within human macrophage cell lines to a level comparable to that of *L. pneumophila*; however, they show varying abilities to replicate within protozoan hosts (26, 45). In addition, non-*pneumophila* species of *Legionella* are less cytotoxic to macrophages, alveolar epithelial cells, and protozoa (3, 26, 29). Although Southern hybridization analysis has revealed that strains of *L. micdadei* and *L. longbeachae* possess *dot*/*icm* homologues, the completeness, functionality, and expression of the type IV secretion system in either species have not been thoroughly examined (3, 24, 29).

In this study, we investigated genetic differences between *L. pneumophila* and *L. micdadei*. Low-stringency genomic subtractive hybridization between a serogroup 1 isolate of *L. pneumophila* and a clinical isolate of *L. micdadei* showed that many known virulence determinants of *Legionella* were *L. pneumophila* specific, including a novel gene involved in the uptake of *L. pneumophila* by host cells which is described in this study.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this study are listed in Table 1. *Legionella* spp. were cultured on BCYE or in ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered yeast extract broth (AYE) (23). When necessary, ampicillin, kanamycin, and chloramphenicol were added to *Legionella* media at 100 μg/ml, 25 μg/ml, and 6 μg/ml, respectively. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) broth or agar aerobically at 37°C. When required, ampicillin, kanamycin, and chloramphenicol were added to bacteriological media at 100 μg/ml, 100 μg/ml, and 12.5 μg/ml, respectively.

Tissue culture conditions and intracellular infection by *Legionella* spp. The human monocytic cell line THP-1 and the human alveolar epithelial cell line A549 were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum, in 5% CO_2 at 37°C. For THP-1 infection, cells were seeded into 24-well tissue culture trays (Sarstedt, Leicestershire, United Kingdom) at a density of 5×10^5 cells/well and pretreated with 10^{-8} M phorbol 12-myristate 13-acetate for 36 to 48 h, in 5% CO_2 at 37°C, to induce differentiation into macrophage-like adherent cells. Stationary-phase *Legionella* cells were added at a multiplicity of infection (MOI) of 5 and incubated for 2 h. Cells were treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria and were subsequently incubated with tissue culture maintenance media. At specified time points, THP-1 cells were washed three times with PBS and treated with 0.1% (vol/vol) Triton X-100 or 0.01% digitonin for lysis and serial dilutions were plated on BCYE agar. For assessment of bacterial uptake, THP-1 lysis and bacterial recovery were performed immediately following gentamicin treatment and washing. Infection of A549 cells was performed as described for THP-1 cells, except that 2×10^5 cells/well were seeded approximately 20 h before infection with *Legionella* using an MOI of 100.

Electron microscopy. THP-1 cells (2×10^6) were differentiated as described above in six-well tissue culture trays (Sarstedt) and infected at an MOI of 10. Infection proceeded for 5 h before cells were washed with phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde for 1 h. Cells were washed in PBS plus 5% sucrose and scraped from the tray surface before being pelleted and washed with PBS plus 5% sucrose followed by a postfixation step in 2.5% osmium tetroxide for 1 h. Following dehydration in a graded acetone series, the cell pellet was embedded in Epon-Araldite epoxy resin. Thin (0.5 μm) sections were stained with 10% uranyl acetate and 2.5% lead citrate before viewing under a Phillips CM12 electron microscope at 60 kV.

General DNA techniques. Bacterial genomic DNA was extracted as described previously (4), and plasmid DNA was isolated using a QIAprep spin Miniprep kit (QIAGEN, Hilden, Germany). DNA-modifying enzymes including restriction endonucleases were used according to the manufacturer's instructions (Promega, Madison, WI). Dot blotting and Southern hybridization were carried out using standard protocols (4). For Southern hybridization, approximately 2 μg of HindIII- and BamHI-digested genomic DNA was separated by agarose gel electrophoresis and transferred to Biodyne PLUS membrane (Pall Corporation, Pensacola, Fla.). Hybridization with labeled probe was performed at 55°C, and membranes were washed under low-stringency conditions (0.5% sodium dodecyl sulfate at 55°C). *L. pneumophila* 02/41 DNA and *L. micdadei* 02/42 DNA were used as positive and negative controls, respectively. Probe DNA was labeled with digoxigen (DIG)–11-dUTP via incorporation during PCR or by random labeling, and DIG detection was performed according to the manufacturers' guidelines (Roche Diagnostics, Mannheim, Germany).

PCR techniques. PCR amplification was performed on 200 ng of template DNA with approximately 0.25 μg of each primer per 25-μl reaction. The reaction conditions were standard with annealing temperatures adjusted for the oligonucleotide melting temperatures. Eleven pairs of oligonucleotides were designed from sequences of known *L. pneumophila* virulence genes for amplification from strains of interest. 5′-GCGATTATTTAAGCAGC and 5′-CTCTCAATCGTAATGAG (annealing temperature, 44°C) yielded a 798-bp product within *ralF*, 5′-GGGTTATCGTGCAAGGC and 5′-GAAGCCAATGCCAAAGG (annealing temperature, 48°C) yielded a 991-bp fragment of *dotA*, 5′-GTATCGCCAAAGCAGCAC and 5′-GAGTTGGCTCATCGGGC (annealing temperature, 50°C) amplified 1,137 bp spanning *dotD-B*, 5′-CCGTCGAGCTTCACTTG and 5′-CAAGACCAGCATCTCCC (annealing temperature, 50°C) yielded a 1,500-bp product within *dotO*, 5′-CACCTCGTGTTAAAGAG and 5′-CATGAACAAAGCGGCTG (annealing temperature, 46°C) amplified 929 bp spanning *icmR-Q*, 5′-CTTCCTCAATCCCCCCC and 5′-CTACACCAGCCTCATCC (annealing temperature, 46°C) yielded a 783-bp product within *plaA*, 5′-CAGGAGTCAGTGTACTTG and 5′-CCAGGTTGTCCTGCTGC (annealing temperature, 50°C) amplified 845 bp spanning *lspF-G*, 5′-CCAATAACCCTTGCCTG and 5′-CGAGTGCTCGTAAAACG (annealing temperature, 48°C) amplified 996 bp spanning *icmV-icmW*, 5′-CGGGCTTTTCCAATGAG and 5′-GTTGGCAGAAAGTACCC (annealing temperature, 48°C) yielded a 1,029-bp fragment within *lvhB4*, 5′-GAAATTGGTGACTGCAGC and 5′-GGGCCATATGCAAGACC (annealing temperature, 48°C) yielded a 612-bp fragment within *mip*, and 5′-GGTGTGCCCGGTTACTC and 5′-GCTTGGCCTCCAGAGTG (annealing temperature, 48°C) amplified a 944-bp product within *rtxA*.

Genomic subtractive hybridization. The PCR-Select bacterial genome subtraction kit (Clontech Laboratories, Palo Alto, CA) was used as described by the manufacturer. Due to the low GC content of *Legionella* spp. and the level of base pair mismatch between *L. pneumophila* and *L. micdadei* (0 to 30%) (42), the temperature of the subtractive hybridization was decreased to 35°C, minimizing the recovery of homologous sequences. Genomic DNA was digested with AluI to generate DNA fragments with a median size of 0.4 kb. DNA fragments were amplified following ligation with specific linkers, cloned into pGEM-T Easy (Promega), and transformed into *E. coli* DH5α competent cells.

Disruption of *lpnE* (lpg2222) in *L. pneumophila* 130b. To generate an insertional mutation, a 1,149-bp fragment of *lpnE* was amplified by PCR with the primers 5′-CGGGATCCATGGACATGAAAAAATATATT and 5′-CCATCGATCTTTTGTCCATTGTCCG (annealing temperature, 42°C) and cloned into pCR-Script. A kanamycin resistance cassette (Km) was then ligated into the native EcoRI site of *lpnE*. This construct was introduced into *L. pneumophila* 130b by natural transformation for homologous recombination as described previously (56). Briefly, bacteria were incubated in AYE broth at 30°C with 10 μg/ml of pCR-Script:*lpnE*::Km until reaching an optical density greater than 1.5 at 660 nm before being spread onto the appropriate CYE plates. Kanamycin-resistant clones were assessed for replacement of *lpnE* with *lpnE*::Km and loss of pCR-Script by PCR and ampicillin sensitivity.

Construction of an *lpnE* transcomplementing vector. To develop a transcomplementation vector with constitutive expression during host cell infection, the 448-bp promoter region of *mip* was cloned into the SacI/XbaI sites of pMMB207, producing pMIP (31). Full-length *lpnE*, including the predicted ribosome binding site, was amplified with 5′-GCTCTAGAGATAGCTCTTAAAAATAAGG and 5′-AACTGCAGGAAAACAGGTAACAGGC (annealing temperature, 44°C) and cloned into the XbaI/PstI sites of pMIP. The resulting plasmid, pMip:*lpnE*, was introduced into *L. pneumophila* 130b *lpnE*::Km via electroporation as described previously (20).

Nucleotide sequencing and analysis. Plasmid DNA was prepared for sequence analysis using a PRISM Ready reaction dye deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The nucleotide sequence of samples was determined by automated DNA sequencing using an Applied Biosystems 3730 DNA analyzer. DNA sequences were assembled using Sequencher 3.1.1 (Gene Codes Corp., MI). BLAST programs were used to determine nucleotide and amino acid homology with sequences in GenBank. The Columbia Genome Center *Legionella* Project (15) and http://genolist.pasteur.fr/LegioList/ (13) were utilized to obtain sequences of entire open reading frames (ORFs) and information on nucleotide sequence surrounding ORFs of interest.

Nucleotide sequence accession number. All nucleotide sequences derived from *L. pneumophila* 02/41 have been submitted to GenBank under accession no. AY688214-29 and AY902807-909.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Serogroup and characteristic(s)	Source or reference
Strains		
L. pneumophila		
130b (ATCC BAA-74)	O1; clinical isolate	22
02/41	O1; environmental isolate (Australia)	MDU[a]
02/40	O1; clinical isolate (Australia)	MDU
03/41	O1; environmental isolate (Australia)	MDU
03/42	O1; environmental isolate (Australia)	MDU
03/43	O1; environmental isolate (Australia)	MDU
03/44	O1; environmental isolate (Australia)	MDU
03/45	O1; environmental isolate (Australia)	MDU
03/46	O1; clinical isolate (Australia)	MDU
03/47	O1; clinical isolate (Australia)	MDU
03/48	O1; clinical isolate (Australia)	MDU
03/49	O1; clinical isolate (Australia)	MDU
03/50	O1; clinical isolate (Australia)	MDU
03/54	O3; environmental isolate (Australia)	MDU
03/55	O4; environmental isolate (Australia)	MDU
03/56	O4; environmental isolate (Australia)	MDU
03/57	O5; environmental isolate (Australia)	MDU
03/59	O6; environmental isolate (Australia)	MDU
03/60	O6; environmental isolate (Australia)	MDU
03/61	O7; environmental isolate (Australia)	MDU
03/63	O8; environmental isolate (Australia)	MDU
03/64	O8; environmental isolate (Australia)	MDU
lpnE::*km*	*lpnE* insertion mutant of 130b (Km^r)	This study
lpnE::*km* (pMIP:*lpnE*)	*lpnE*::*km* carrying pMIP:*lpnE* (Km^r Cm^r)	This study
L. micdadei		
02/42	Clinical isolate (Australia)	MDU
02/43	Quality assurance standard (Australia)	MDU
03/67	Environmental isolate (Australia)	MDU
L. longbeachae		
ATCC 33462	O1; type strain (U.S.)	37
A5H5	Clinical isolate (Australia)	19
A4C5	Clinical isolate (Australia)	19
D-1750	Clinical isolate (U.S.)	19
D-493	Clinical isolate (U.S.)	19
Atlanta-5	Clinical isolate (U.S.)	19
D-880	Clinical isolate (U.S.)	19
LA-24	Clinical isolate (U.S.)	19
L6C9	Environmental isolate (Australia)	19
K8B9	Environmental isolate (Australia)	19
L. gormanii 03/69	Environmental isolate (Australia)	MDU
L. jordanis 03/70	Environmental isolate (Australia)	MDU
E. coli DH5α	F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Clontech
Plasmids		
pCR-Script	High-copy cloning vector (Amp^r)	Stratagene
pGEM-T Easy	3,015-bp cloning vector	Promega
pMMB207	RSF1010 derivative; IncQ *oriT lacI*^{q} P*tac* Cm^r	41
pMip	pMMB207 with the promoter region of *mip* cloned into *Sac*I/*Xba*I	This study
pMip:*lpnE*	pMip carrying *lpnE* and ribosome binding site, cloned into *Xba*I/*Pst*I	This study

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RESULTS

Characterization of *L. pneumophila* and *L. micdadei* strains for subtractive hybridization. The intraspecies genetic heterogeneity among isolates of *L. pneumophila* is not well documented and even less well investigated for *L. micdadei*. Thus, to examine broadly the suitability of *L. pneumophila* and *L. micdadei* strains in our collection for subtractive hybridization, we tested two serogroup 1 *L. pneumophila* strains (02/40 and 02/41) and two strains of *L. micdadei* (02/42 and 02/43) for the presence of several known virulence-associated loci of *L. pneumophila*.

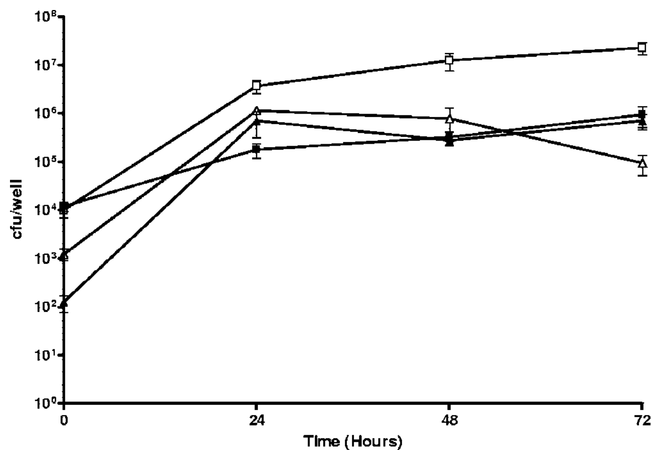

FIG. 1. Replication of *L. pneumophila* strain 02/41 (▲ and △) and *L. micdadei* strain 02/42 (■ and □) in THP-1 macrophages (open) and A549 alveolar epithelial cells (solid). Results are expressed as the number of cell-associated bacteria and are the mean ± standard deviation of at least three independent experiments from duplicate wells.

These were *ralF*, *dotA*, *dotD-B*, *dotO*, *icmR-Q*, *plaA*, *lspF-G*, *icmV-W*, *lvhB4*, *mip*, and *rtxA*. *L. pneumophila* 02/41 was positive for all virulence loci by PCR, while *L. pneumophila* 02/40 was negative by PCR for *ralF*, *dotA*, and *icmV-W* (data not shown). In contrast, while both *L. micdadei* 02/42 and 02/43 were positive by PCR for *lvhB4*, these strains were negative by PCR for all other loci tested (data not shown). *L. pneumophila* 02/41 and *L. micdadei* 02/42 were then chosen for further comparison by genomic subtractive hybridization.

To examine any phenotypic differences between *L. pneumophila* 02/41 and *L. micdadei* 02/42 in their ability to survive within macrophages and epithelial cells, these strains were tested for their ability to replicate within the human macrophage-like cell line THP-1 and in A549 human alveolar epithelial cells, as well as for their ability to recruit RER to the replicative vacuole. As expected, both strains were able to replicate within macrophages and epithelial cells (Fig. 1); however, *L. pneumophila* 02/41 recruited RER to the vacuole 5 h after infection of THP-1 cells (Fig. 2A), whereas the *L. micdadei* 02/42 vacuole showed no association with RER at the same time point (Fig. 2B). Of the 10 individual THP-1 cells examined that were infected with *L. pneumophila* 02/41, all LCVs showed an association with RER. In contrast, none of the 10 individual THP-1 cells examined that were infected with *L. micdadei* 02/42 contained LCVs that were associated with RER.

Genomic subtractive hybridization between *L. pneumophila* 02/41 and *L. micdadei* 02/42. To determine the appropriate stringency for subtractive hybridization of *L. pneumophila* 02/41 with *L. micdadei* 02/42, we tested a range of hybridization temperatures, including 35°C, 45°C, and 55°C. Low-stringency conditions were used to ensure homologous sequences were subtracted, allowing for the average G+C content (38%) of *L. pneumophila* and up to 30% reported base pair mismatch between *L. pneumophila* and *L. micdadei* (42). The specificity of the PCR products obtained following subtractive hybridization was examined by low-stringency Southern hybridization of subtracted *L. pneumophila* 02/41 DNA probed with DIG-labeled *L. micdadei* genomic DNA. Subtracted DNA fragments amplified from the hybridization conducted at 35°C showed the least reactivity with *L. micdadei* DIG-labeled DNA (Fig. 3), and these products were subsequently cloned into pGEM-T Easy. A total of 152 clones were recovered, and these were tested by dot blot hybridization for reactivity with *L. pneumophila* 02/41 and *L. micdadei* 02/42 DIG-labeled genomic DNA. Eight clones (5.3%) hybridized with both *L. pneumophila* 02/41 and *L. micdadei* 02/42 genomic DNA and were classified as nonspecific. The remaining 144 clones only hybridized with *L. pneumophila* 02/41 DNA.

Identification of *L. pneumophila* 02/41-specific sequences. Sequencing of the 144 *L. pneumophila*-specific clones showed an average clone size of 375 bp representing 151 putative and known *L. pneumophila* ORFs (see Table S1 in the supplemental

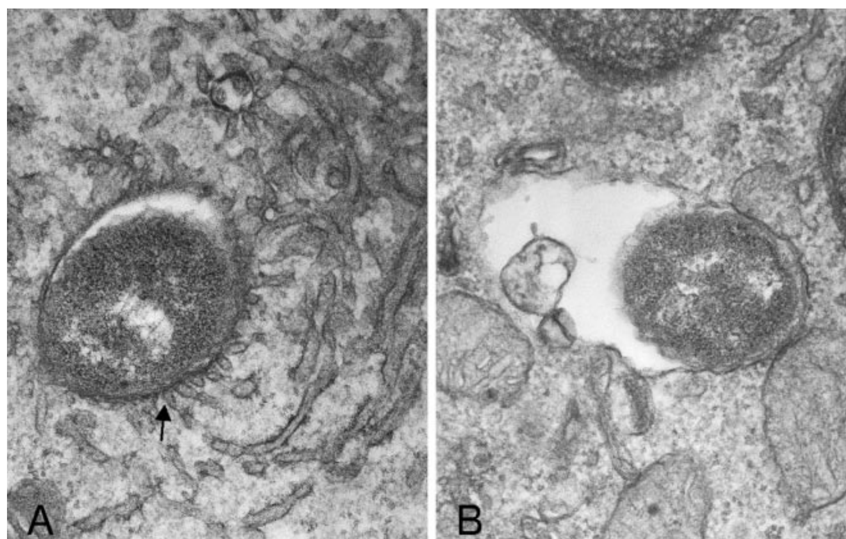

FIG. 2. Transmission electron microscopy of THP-1 cells infected with *L. pneumophila* strain 02/41 and *L. micdadei* strain 02/42. (A) 02/41. Magnification, ×45,000. An arrow indicates fusion of RER to the LCV. (B) 02/42. Magnification, ×45,000.

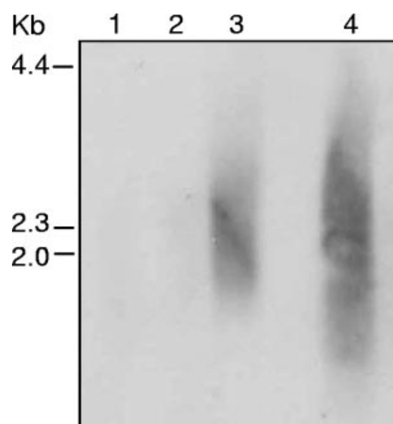

FIG. 3. Southern hybridization of subtracted and PCR-amplified *L. pneumophila* 02/41 DNA fragments probed with randomly labeled AluI-digested *L. micdadei* 02/42 genomic DNA. Equal amounts of subtracted DNA were loaded in each lane. Lane 1, subtraction performed at 35°C; lane 2, subtraction performed at 45°C; lane 3, subtraction performed at 55°C; lane 4, AluI-digested *L. micdadei* 02/42 genomic DNA.

material). Twenty-two clones were absent in at least one of the three sequenced *L. pneumophila* genomes, with only 1, clone 2B6, appearing to be strain specific, with no homologous nucleotide sequence in any of the sequenced *L. pneumophila* genomes (13, 15). This level of conservation between the three *L. pneumophila* genome sequences and *L. pneumophila* strain 02/41 supports the hypothesis that genes intrinsic to the function and virulence of *L. pneumophila* serogroup 1 may be identified by genomic comparisons with similar and less virulent *Legionella* spp. Further sequence analysis of the clones showed that 36% corresponded to hypothetical proteins and ORFs with no significant homologues and no known function. The remaining clones had a range of predicted functions and included 16 ORFs with known or putative roles in virulence, 10 putative regulators, and 13 genes putatively involved in cell wall and lipopolysaccharide (LPS) biosynthesis (see Table S1 in the supplemental material).

Distribution of selected sequences among *L. pneumophila* of different serogroups and other *Legionella* spp. Of the 144 clones sequenced, 41 different sequences representing 40 ORFs were selected to cover a range of putative functions for testing by low-stringency Southern hybridization to examine their distribution among *L. pneumophila* strains of different serogroups as well as additional strains of *L. micdadei*, *L. longbeachae*, *L. gormanii*, and *L. jordanis*. Overall, the sequences fell into four broad groups. Twelve different ORFs (30%) were present only in *L. pneumophila* (group I, Table 2); a further 9 ORFs (22.5%) were present in all *L. pneumophila* strains tested and an isolate of *L. gormanii* (group II, Table 2); 15 ORFs (37.5%) were present in most or all strains of *L. pneumophila*, *L. longbeachae*, *L. gormanii*, and/or *L. jordanis* (group III, Table 2); and 4 ORFs (10%) were present in all *Legionella* spp., including *L. jordanii* and *L. micdadei* (group IV, Table 2).

The 12 different ORFs in group I included 2 genes identified previously, *ladC* and *lepB* (14, 47), 2 putative transcriptional regulators, 2 putative zinc-dependent protease genes, including one with homology to virulence-associated elastases (9); and 4 ORFs predicted to play a role in lipopolysaccharide and cell wall biogenesis. Two clones, E10 and 2B5, were found within a gene predicted to encode an N-acetylmuramoyl-L-alanine amidase. Autolysins are common among different bacterial species and are associated with various cellular processes such as cell growth, cell-wall turnover, motility, protein secretion, differentiation, biofilm

TABLE 2. Distribution of selected sequences among strains of *L. pneumophila*, *L. gormanii*, *L. longbeachae*, *L. jordanis*, and *L. micdadei*

Clone name[a]	Corresponding *L. pneumophila* Philadelphia gene(s) or locus tag	Known or putative function
Group I		
2B7, B11, C12/F3, F8[b]	*yvf*, lpg0754, *waaM*, lpg0774	LPS and lipidA biosynthesis
E10/2B5	lpg2699	Autolysin
2E6	*lepB*	Dot/Icm effector
2C12	lpg2222	Hypothetical TPR repeat protein
G7	*ladC*	Adenylate cyclase
C3, B5	lpg2977, lpg1176	Zn metalloproteases
D12, E12	*pleD*, lpg1357	Transcriptional regulators
Group II		
D6	*icmD*	Dot/Icm secretion system
C10	*pilM, pilN*	Type IV pilus biosynthesis
2C5	*clpB*	Heat shock chaperone
C1/2C8	lpg0919	Zn metalloprotease
A8	*enhA*	Unknown function
A3/B2	*cadA2*	Cadmium efflux ATPase
H1	lpg2549	Transcriptional regulator
Group III		
B8	*rep*	ATP-dependent DNA helicase
A6	*icmE*	Dot/Icm secretion system
C2	*lspI, lspJ*	Type II secretion system
H7/2C6, C6, F5/F6[c]	lpg0142, lpg1071, lpg2119	Transposases
D1	lpg1292, lpg1291	Two-component response regulator
F11	*hisF*, *hisH*	Amino acid metabolism
C9[d]	*sidH*	Dot/Icm effector
E6[e]	*sidE*	Dot/Icm effector
2C11[f]	*sidB*	Dot/Icm effector
2E3[g]	*sidG*	Dot/Icm effector
Group IV		
A11,[h] E2,[i] G12[j]	lpg0991, lpg0986, lpg0987	Hypothetical proteins
2E10	*udk*, fabI	Lipid metabolism

[a] Group I was present only in *L. pneumophila.* Group II was present only in *L. pneumophila* and *L. gormanii.* Group III was present in *L. pneumophila, L. longbeachae, L. gormanii*, and/or *L. jordanis.* Group IV was found in all species examined. Full details of all clones are provided in Table S1 in the supplemental material.

[b] Not detected in *L. pneumophila* strains 03/54, 03/56, 03/60, 03/61, 03/63, and 03/64.

[c] Not detected in *L. pneumophila* strain 03/61.

[d] Not detected in *L. pneumophila* strains 03/41, 03/54, and 03/61 or *L. longbeachae* strains A4C5, D-493, and LA-24. Present in *L. gormanii* strain 03/70 and *L. jordanis* strain 03/69.

[e] Not detected in *L. pneumophila* strains 02/40 and 03/47, *L. longbeachae* strain K8B9, or *L. jordanis* strain 03/69. Present in *L. gormanii* strain 03/70.

[f] Not detected in *L. longbeachae* strain D-1750 and ATCC. Present in *L. gormanii* strain 03/70 and *L. jordanis* strain 03/69.

[g] Not detected in *L. pneumophila* strain 02/40 or *L. jordanis* strain 03/69.

[h] Not detected in *L. longbeachae* strain A5H5 or *L. jordanis* strain 03/69.

[i] Not detected in *L. longbeachae* strains A5H5, L6C9, and ATCC or *L. jordanis* strain 03/69.

[j] Not detected in *L. longbeachae* strain A5H5 or *L. jordanis* strain 03/69.

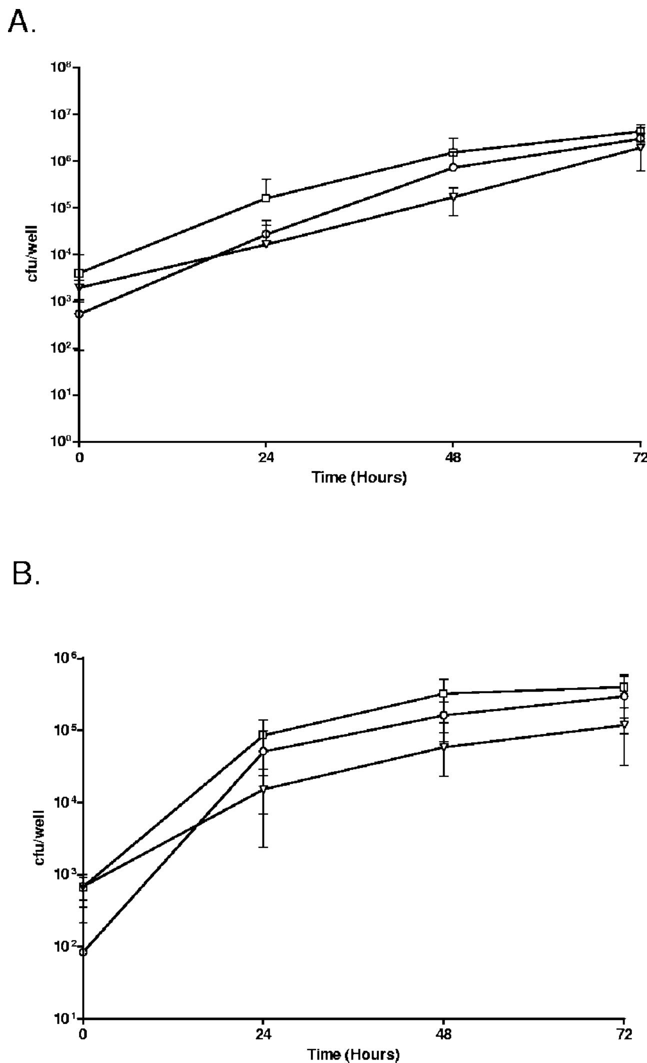

FIG. 4. Replication of derivatives of *L. pneumophila* strain 130b in THP-1 macrophages (A) and A549 alveolar epithelial cells (B). *L. pneumophila* 130b (□), *lpnE*::*km* (○) and *lpnE*::*km* (pMIP:*lpnE*) (▼). Results are expressed as the number of cell-associated bacteria and are the mean ± standard deviation of at least three independent experiments from duplicate wells.

formation, and pathogenicity (10, 11, 58). The eight ORFs present in group II comprised a putative transcriptional regulator and several genes previously identified in *L. pneumophila*, including *cadA2*, *icmD*, and *ceaA*, of which *cadA2* and *ceaA* have been shown to be upregulated during intracellular growth (47). Other genes within this group included a homolog of *enhA*, the 5′ end of the *pilMNOPQ* locus, a putative Zn-dependent protease gene, and a homolog of *clpB*. Of the 15 ORFs in group III, clone D1 showed homology to a novel, intact two-component response regulator. The closest homologues of this response regulator comprise a group of DNA binding proteins highly homologous to CheY-like receiver domains involved in bacterial chemotaxis (21). Group III also included four ORFs, *sidB*, *sidE*, *sidG*, and *sidH*, which encode secreted substrates of *dot*/*icm* type IV secretion system which were identified using an interbacterial protein transfer technique (34). Group IV contained 4 ORFs, of which clones A11, G12, and E2 are clustered in a region of hypothetical and unknown ORFs on the basis of the *L. pneumophila* genome sequences. Clones A11, E2, G12, and 2E10 were the only sequences present in the two additional strains of *L. micdadei* tested, suggesting that their absence in the subtraction driver strain, *L. micdadei* 02/42, may be strain specific.

Characterization of an *L. pneumophila*-specific gene encoding a putative protein with eight tetratricopeptide repeats. One of the putative virulence-associated genes present in group I included the locus tag lpg2222, which is predicted to encode a protein that shares similarity with EnhC. EnhC has been shown to play a role in enhancing the uptake of *L. pneumophila* into host cells (16). To determine if lpg2222 also contributes to the entry of *L. pneumophila* into host cells, we inactivated lpg2222 by insertion of a kanamycin resistance cassette. The resulting mutant showed no significant defect in intracellular replication during *L. pneumophila* infection of both the THP-1 and A549 cell lines (Fig. 4A and B). However, the lpg2222 mutant did exhibit reduced entry into THP-1 and A549 cells compared with wild-type *L. pneumophila* 130b, which was partially restored upon *trans*-complementation of the mutant with pMIP:*lpnE* (Table 3). Interestingly, although pMIP:*lpnE* was able to complement the entry defect, carriage of the plasmid appeared to interfere with intracellular replication of the *trans*-complemented lpg2222 mutant. pMIP:*lpnE* is derived from the IncQ plasmid, pMMB207, and previous studies have reported that pMMB207 can interfere with intracellular replication of *L. pneumophila* (51, 54). This defect has been attributed to the presence of mobilization factors on the plasmid that presumably interfere with Dot/Icm function (54), and this is likely to be the reason for the replication defect observed here.

The decrease in entry observed for the lpg2222 mutant was not the result of decreased attachment to host cells as the lpg2222 mutant demonstrated levels of attachment to both host cell lines similar to those of wild-type *L. pneumophila* 130b. Bacterial attachment was calculated as the percentage of the original inoculum that remained after infection for 2 h. In infected THP-1 cells, the percentages of cell-associated bacteria were 1.37% ± 0.70% for *L. pneumophila* 130b and 1.12% ± 0.71% for the lpg2222 mutant. Similarly the percentage of the inoculum associated with A549 cells for 130b was 0.50% ±

TABLE 3. *L. pneumophila* entry into THP-1 and A549 cell lines

Strain	% of intracellular bacteria in[a]:	
	THP-1	A549
130b	0.40 ± 0.24	0.0063 ± 0.0036
lpnE::*km*	0.09 ± 0.07[b]	0.0004 ± 0.0006[b]
lpnE::*km* (pMip:*lpnE*)	0.21 ± 0.12[c]	0.0046 ± 0.0022[c]

[a] Gentamicin-resistant bacteria as a percentage of the inoculum. Results are means ± standard deviations from at least three independent experiments in duplicate wells.

[b] Significantly less than values for *L. pneumophila* 130b ($P < 0.01$ by an unpaired two-tailed t test).

[c] Significantly greater than *L. pneumophila* *lpnE*::*km* ($P < 0.05$ by an unpaired two-tailed t test).

0.24%, and for the lpg2222 mutant it was 0.58% ± 0.29%. In light of these results, lpg2222 was renamed *lpnE* for "gene involved in *L. pneumophila* host cell entry."

DISCUSSION

Outbreaks of Legionnaires' disease worldwide are almost exclusively due to infection with *L. pneumophila*, and this species is also responsible for the vast majority of sporadic cases of legionellosis (35). Although infection with non-*pneumophila Legionella* species can occur (43), overall, non-*pneumophila* species account for around only 10% of cases of legionellosis (59). While environmental factors and genetic regulation may contribute to this epidemiological difference, it is widely believed that *L. pneumophila* possesses species-specific virulence determinants that account for its greater prominence as a pathogen.

In this study, we identified specific genetic differences between a serogroup 1 strain of *L. pneumophila* and an isolate of *L. micdadei*. In the absence of a genome sequence for *L. micdadei*, the genetic comparison was done experimentally by genomic subtractive hybridization (1). Of the resulting 144 *L. pneumophila* 02/41-specific clones, many were genes with unknown function, but some known and putative virulence determinants were also identified as absent in *L. micdadei*.

Forty-one sequences, corresponding to 40 different predicted ORFs in the *L. pneumophila* Philadelphia, Paris, and Lens genome sequences (13, 15) covering a range of functions, were examined for their prevalence among various serogroups of *L. pneumophila* and other species of *Legionella* by low-stringency Southern hybridization. The results showed that 30% of these clones were *L. pneumophila* specific, and a further 22.5% were present in *L. pneumophila* and *L. gormanii* only.

Several genes involved in cell wall biosynthesis were identified in this study, including five genes within the *L. pneumophila* LPS biosynthesis gene locus. Clones 2B7 and B11, representing neighboring *orf21* (*lyrF*) and *orf22* (lpg0754), were *L. pneumophila* specific. This piece of data is consistent with a recent hybridization study showing that a probe encompassing *orf21* to *orf26* of the LPS biosynthesis locus hybridized to *L. pneumophila* of serogroups 2 to 14 but showed no hybridization to a range of non-*pneumophila Legionella* species (33). In this study we also showed that clone F11, spanning *orf26* (*hisF*) and *orf27* (*hisH*), hybridized to all strains of *L. longbeachae* and *L. gormanii*, suggesting that *orf27* is shared among different species of *Legionella*. In addition, we found that clone F8, corresponding to *orf3* (lpg0774) of the LPS biosynthesis locus, was present in all serogroup 1 strains of *L. pneumophila* as well as serogroups 4, 5, and 6. This distribution differs from that found by Luneberg et al., who reported that a probe spanning *orf1* to *orf4* hybridized only to serogroups 1 and 7 of *L. pneumophila*. This discrepancy suggests that the distribution of *orf3* is highly variable among different *Legionella* species and strains.

A selection of known *L. pneumophila* virulence loci were also identified by this subtractive hybridization study. These included two members of the *dot*/*icm* type IV secretion system, *icmD* and *icmE*, and five genes that encode Dot/Icm effector proteins that are translocated into host cells, *lepB*, *sidB*, *sidE*, *sidG*, and *sidH* (14, 34). *lepB* encodes a putative SNARE homologue involved in nonlytic release of *L. pneumophila* from *Dictyostelium discoideum* that may contribute to the transmission of *L. pneumophila* by promoting the egress of bacteria from amoebae (14). Similar to *ralF*, which encodes another Dot/Icm effector, *lepB* was present only in strains of *L. pneumophila* (44). Interestingly, the same was not true for *sidB*, *sidE*, *sidG*, and *sidH*, which, as well as being present in all *L. pneumophila* serogroups tested, were found in most strains of *L. longbeachae*, *L. jordanis*, and *L. gormanii* used in this study. *icmE* (*dotG*) was present in *L. gormanii* and all *L. longbeachae* strains but absent in *L. micdadei* and *L. jordanis*. Conversely, *icmD* was found in *L. pneumophila* and *L. gormanii* but none of the other non-*pneumophila* species examined. A previous study investigating the distribution of *icmD* and *icmE* genes among different species of *Legionella* also showed that both loci were absent in *L. micdadei* and *L. jordanis* (42). Recently, species-specific heterogeneity was described for the *icmR* locus in *L. pneumophila*, *L. longbeachae*, and *L. micdadei*. The gene occupying this position in each species was unrelated by amino acid similarity but performed the same function as a chaperone for its cognate IcmQ protein (24). Overall, these data suggest that not only do the *dot*/*icm* loci vary in their organization and composition among different *Legionella* strains and species but that some Dot/Icm effector proteins, such as RalF and LepB, may be species specific (14, 44).

In this study we also identified three sequences corresponding to *L. pneumophila* genes expressed during replication within U937 macrophages (47). Of these, *ladC* encodes a putative adenylate cyclase which was *L. pneumophila* specific (group I), whereas *cadA2* and *ceaA* were found in all *L. pneumophila* strains and in *L. gormanii* but were absent from all of the other species tested (group II). *cadA2* and *ceaA* encode a putative cadmium efflux pump and a component of a chemiosmotic efflux system, respectively. Overall, 9.3% of ORFs identified in this study represent genes predicted to be involved in transport and efflux of various ions. The large number and apparent functional redundancy of dedicated transport systems in *L. pneumophila* have been described previously and may reflect the importance of detoxification and ion balance during intracellular replication of *L. pneumophila* (36, 47).

Among other group II sequences, we identified the 5′ region of the *pilMNOPQ* locus. The *pil* locus of *L. pneumophila* is essential for type IV pilus biogenesis, but only the prepilin peptidase encoded by *pilD* is also essential for type II protein secretion, intracellular infection, and virulence in A/J mice (32, 51). Interestingly we also identified *lspJ*, which is predicted to encode a pseudopilin of the Lsp type II secretion system. The Lsp type II protein secretion apparatus secretes a number of enzymes, including the zinc metalloprotease, ProA, and is required for growth of *L. pneumophila* in both *Hartmannella vermiformis* and *Acanthamoeba castellanii* (28, 50). A recent study has also shown that the Lsp system is essential for virulence in the A/J mouse model of infection (28, 46, 50, 51), implying an important role in virulence factor export. While this gene was absent in *L. micdadei* and *L. jordanis*, it was present in all strains of *L. pneumophila*, *L. longbeachae*, and *L. gormanii*.

Several unknown ORFs specific for *L. pneumophila* or *L. pneumophila* and *L. gormanii* were identified in this study that may represent novel virulence determinants. Three of these showed homology to proteases from other pathogens, including vibrolysin and elastases, which are known to contribute to the

virulence of *Vibro vulnificus*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila*, respectively (12, 18, 38). In addition, we identified an *L. pneumophila*-specific ORF, locus tag lpg2222, with predicted similarity to the product of *enhC*. *enhC* is part of a locus involved in *L. pneumophia* entry into host epithelial cells and macrophages (16). Further characterization of lpg2222, termed *lpnE*, demonstrated that this gene was also required for full entry of *L. pneumophila* into THP-1 and A549 cells. This finding is consistent with the function of EnhC in enhancing *Legionella* entry presumably through the possession of multiple tetratricopeptide repeats (16). These repeat regions are associated with a range of functions in eukaryotic cells through their ability to mediate protein-protein interactions (27), and we predict that these regions may be important for LpnE-mediated interaction of *L. pneumophila* with host cells. Recently, the product of *lpnE* was identified by proteomic analysis as a protein upregulated during *L. pneumophila* growth in medium designed to induce resistance to stress, termed ERS (enhanced resistance to stress) treatment (5). The authors found that ERS treatment of *L. pneumophila* promoted Dot/Icm-independent entry into macrophages and amoebae, demonstrating that there was a correlation between *lpnE* expression and levels of bacterial uptake into host cells.

In summary, this study has identified distinct genetic differences between *L. pneumophila* and other species of *Legionella*. These differences encompassed many known and putative virulence determinants that may contribute to the increased virulence and unique intracellular lifestyle of *L. pneumophila.* Further work will help to define the importance of this genetic diversity to the pathogenesis of infections with both *L. pneumophila* and non-*pneumophila* species.

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