Legionella steelei sp. nov., isolated from human respiratory specimens in California, USA, and South Australia

Paul H. Edelstein,¹ Martha A. Edelstein,¹ Lisa J. Shephard,² Kevin W. Ward³ and Rodney M. Ratcliff^{2,4}

Correspondence

Paul H. Edelstein
phe@mail.med.upenn.edu

¹University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

²Microbiology and Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, SA Pathology, Adelaide, Australia

³UCLA Health System Clinical Laboratories–Microbiology, Los Angeles, CA, USA

⁴School of Biomedical Science, The University of Adelaide, Adelaide, Australia

Legionella-like bacteria were isolated from the respiratory tract of two patients in California, USA, and South Australia, but were not thought to cause disease. These bacteria, strains F2632 and IMVS-3376^T, were found to have identical *Legionella* macrophage infectivity potentiator (*mip*) gene sequences and were therefore further characterized to determine their genetic and phenotypic relatedness and properties. Both of these Gram-negative-staining bacterial strains grew on buffered charcoal yeast extract medium, were cysteine auxotrophs and made a characteristic diffusible bright yellow fluorescent pigment, with one strain making a late appearing colony-bound blue–white fluorescent pigment. The optimal *in vitro* growth temperature was 35 °C, with very poor growth at 37 °C in broth or on solid media. There was no growth in human A549 cells at either 35 or 37 °C, but excellent growth in *Acanthamoeba castellanii* at 30 °C and poorer growth at 35 °C. Phylogenetic analysis of these bacteria was performed by sequence analysis of 16S rRNA, *mip*, ribonuclease P, ribosomal polymerase B and zinc metalloprotease genes. These studies confirmed that the new strains represented a single novel species of the genus *Legionella* for which the name *Legionella steelei* sp. nov. is proposed. The type strain is IMVS-3376^T (=IMVS 3113^T=ATCC BAA-2169^T).

Members of the genus *Legionella* are Gram-negative bacteria that can cause a type of pneumonia termed Legionnaires' disease and rarely non-pneumonic infectious diseases (Edelstein & Cianciotto, 2010). *Legionella pneumophila* is the primary cause of Legionnaires' disease and 19 other species of the genus *Legionella* have been shown to cause the disease, mostly in immunocompromised patients. At the time of writing, there were 53 members of the genus *Legionella* with validly published names, 33 of which have been isolated only from environmental sources (<http://www.bacterio.cict.fr/index.html>). All of the recognized species of the genus *Legionella* that have been examined for intracellular growth have been found to be facultative or obligate intracellular parasites of

free living amoebae, and those causing human disease are facultative intracellular parasites of human cells (Cianciotto *et al.*, 2006).

Identification of members of the genus *Legionella* was originally based on DNA–DNA hybridization, supplemented by serological identification, cellular fatty acid profiles and ubiquinone synthesis and other phenotypic tests (Brenner, 1987). However, serological cross-reactivity and the non-availability of antibodies to many recently described species makes serotyping a difficult and frequently a non-specific method of species identification. DNA–DNA hybridization is performed at only a few laboratories worldwide. Finally, cellular fatty acid and ubiquinone analyses are rarely performed for species identification, as typical patterns are shared for some species and are unavailable for several recently described *Legionella* species. Genomic DNA sequence analysis based on multiple gene targets has become the standard for the identification of members of the genus *Legionella* because of its portability, specificity and ease of performance (Lück *et al.*, 2010).

Abbreviation: *mip*, macrophage infectivity potentiator.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *mip*, *proA*, *rnpB* and *rpoB* gene sequences of strain IMVS-3376^T are HQ398202–HQ398206, respectively.

Two supplementary figures are available with the online version of this paper.

In this study, the discovery of a novel species of the genus *Legionella* is described. The novel species was found following an examination of *Legionella*-like bacteria from the respiratory tracts of two patients from different continents who were thought not to have Legionnaires' disease. Independent identification of these clinical isolates by macrophage infectivity potentiator (*mip*) gene sequencing led to further testing to determine whether these two strains represented a single novel species.

Methods

Phenotypic testing. Bacteria were grown on laboratory-made MOPS buffered charcoal yeast extract medium supplemented with α -ketoglutarate (BCYE α agar), ACES buffered yeast extract broth (BYE α broth) and ACES buffered charcoal yeast extract broth (BCYE α broth), and incubated in humidified incubators with or without 5% CO $_2$ for plates, or in shaking incubators for broth cultures (Edelstein & Edelstein, 1991, 1993). Tests for growth on selective media used commercially prepared BCYE α media with added polymyxin B, anisomycin and cefamandole (BBL PAC; BD Diagnostics), or with added polymyxin B, anisomycin and vancomycin (BBL PAV). To detect flagella, bacteria were grown on a BCYE α plate at 35 °C, then harvested into sterile water. The suspended bacteria were kept at room temperature for two to five days and then stained for flagella using the Ryu stain (Kodaka *et al.*, 1982). Bacterial motility in amoebae was determined by microscopic examination of intra-amoebic bacteria. Gram reaction, oxidase, gelatinase and hippurate reactions, and glucose utilization were determined as described previously (Edelstein *et al.*, 1982). Diffusible and colony fluorescence of plate-grown bacteria was detected by illumination with a long wave UV light in the dark. Reactivity with direct fluorescent antibodies to several different *Legionella* species was tested as described previously (Edelstein, 2004). Determination of the presence of immunoreactive *L. pneumophila* DotA and IcmX was conducted as previously described (Higa & Edelstein, 2001). Analysis of cellular fatty acid composition by gas liquid chromatography of fatty acid methyl esters was performed by Midi Laboratories using 24 h growth on heavily inoculated BCYE α plates incubated in humidified ambient air at 35 °C (Sherlock version 6.1, Midi Laboratories, Inc.).

The ability of the bacteria to grow in amoebae was determined by inoculating the plate-grown (35 °C) bacteria into tissue culture plates containing axenic *Acanthamoeba castellanii* (ATCC 30234) in autoclaved dechlorinated tap water as described previously (Boulanger & Edelstein, 1995). The bacterial-amoebal co-cultures were incubated at 30 °C in ambient air for up to seven days and followed microscopically and by supernate cultures placed onto BCYE α plates incubated at 35 °C. Control strains included *L. pneumophila* serogroup 1 strain F889, a human isolate that is highly virulent for amoebae, human and guinea pig macrophages and guinea pigs (Edelstein *et al.*, 1987); and F2352, a non-virulent *L. pneumophila* dotA mutant.

Growth of the bacteria in the human respiratory alveolar epithelial cell line A549 was studied in tissue culture wells incubated at 35 °C as described previously (Higa & Edelstein, 2001). Intracellular growth was monitored both microscopically and by viable plate counts.

Genotypic analysis. Crude bacterial DNA preparations were made by boiling heavy suspensions from plate-grown bacteria in EDTA (5 mM) or in Tris/HCl (10 mM) and EDTA (1 mM) (TE) for 10 min. The boiled supernatant was used in polymerase chain reactions (PCRs) and PCR products were sequenced on both strands by standard cycle sequence methods, as previously described (Ratcliff *et al.*, 1998). Primers and amplification conditions were as previously described for: macrophage infectivity potentiator (*mip*) (Ratcliff *et al.*, 1998); ribonuclease P (*rnpB*) (Rubin *et al.*, 2005); and ribosomal polymerase B (*rpoB*) (Ko *et al.*, 2002). Small subunit rRNA gene (16S rRNA) used primers Leg-16S-F (5'-GGCTCAGATTGAACGCTGGCGG-3') and Leg-16S-R (5'-ACCCACTCCCATGGTGTGACGG-3') using 40 cycles of 94 °C (30 s), 55 °C (1 min) and 72 °C (2 min); additional sequencing primers used were Leg16S-Int-R (5'-TTTACGCCCAGTAATTCCG-3'), LEG16s-Int-F (5'-CAGCAGCCGCGGTAATACG-3') and Leg16S-Int2-F (5'-TGT-CGTGCTCGTGTCTGTG-3'); zinc metalloprotease (*proA*) used LegProA-F (5'-TGCATTTRTAYGCNGGNTAYGTNAT-HAARCAYATGTA-3') and LegProA-R (5'-GTGTCCARTARTCCATRTTNGCYTTNACCAT-3') with 60 cycles of 94 °C (30 s), 50 °C (1 min) and 72 °C (2 min).

Sequence assembly, alignment and analysis were performed using Kodon (Applied Maths) and the phylogenetic analysis (maximum-likelihood) was performed using PAUP 4 (Sinauer Associates). Modeltest 3.7 (Posada & Crandall, 1998) was used to assess the best-fit model (GTR+I+G) used for the analysis and relationship trees were drawn with FigTree v.1.1.2 (Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh, UK, <http://tree.bio.ed.ac.uk/software/figtree/>). Bootstrap analysis (1000 replicates) of the maximum-likelihood dendrogram was performed using RAxML accessed through the web-based CIPRES (Miller *et al.*, 2010) portal (<http://www.phylo.org/portal2>). SplitsTree analysis was performed using SplitsTree4 (Huson & Bryant, 2006; <http://www.splitsree.org/>).

Sequences of isolate IMVS-3376^T were deposited in GenBank/EMBL/DDBJ with accession numbers HQ398202 (16S rRNA), HQ398203 (*mip*), HQ398204 (*proA*), HQ398205 (*rnpB*) and HQ398206 (*rpoB*).

Results

Case histories. Patient 1, an adult male, who was previously healthy, had suspected bronchitis in February 1999, in Adelaide, Australia. Cultures for *Legionella* were performed from sputum. A slowly growing legionella-like organism was isolated at the Microbiology and Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, Australia. The patient was thought not

to have a legionella infection based on clinical grounds. Of note was the fact that the patient had been making and using compost for gardening prior to the onset of his bronchitis. The bacterial isolate was designated isolate number IMVS-3376^T. The isolate appeared to be a novel species based on the absence of reactivity with available antisera as well as *mip* gene sequence analysis.

Patient 2, an adult male, had suspected pneumonia in September 2008, in Los Angeles, California, USA, while hospitalized for severe congestive heart failure. Bronchoscopy revealed a blood clot in the left main bronchus. Culture of a bronchoalveolar lavage specimen at the UCLA Health System Microbiology Laboratory, USA, grew a legionella-like bacterium on BCYE α medium at 35 °C in humidified air after four days incubation. Consultants felt that the patient was unlikely to have Legionnaires' disease but treated the patient regardless with a variety of antimicrobials known to be active against *L. pneumophila*. The patient died several months later of his underlying disease. Identification of the bacterial isolate by 16S rRNA sequencing (500 bp) revealed 96% sequence similarity to *Legionella anisa*. The isolate was referred to a research laboratory at the Hospital of the University of Pennsylvania, USA, for definitive identification and was given isolate number F2632. The isolate appeared to represent a novel species based on absence of reactivity with available antisera; *mip* gene sequencing revealed the similarity of the strain to the corresponding gene sequence of isolate IMVS-3376^T.

Phenotypic characterization. Both strains IMVS-3376^T and F2632 were characterized in one laboratory (Philadelphia, USA) to determine their phenotypic properties. Both isolates were Gram-negative-staining rods that were cysteine auxotrophs. They both possessed a single monopolar flagellum and grew on BCYE α medium in ambient air.

The bacterial colonies were round, 0.5–2 mm in diameter, entire and convex, with an opalescent ground glass appearance when examined using a dissecting microscope. A large amount of a diffusible yellow–green fluorescent pigment was produced by the bacteria growing on BCYE α medium, such that the entire plate appeared yellow when illuminated by long wave UV light. In addition, individual colonies of strain IVMS-3376^T, but not strain F2632, were brightly electric blue–white fluorescent; this fluorescence was not diffusible into the surrounding medium and appeared to be maximal for isolated colonies and suppressed when there was confluent bacterial growth. Both isolates grew well on the selective BCYE α media PAC and PAV at 35 °C.

Neither isolate utilized glucose and both were oxidase-, hippurate-, catalase-, β -lactamase- and gelatinase-positive. There were no serological reactions with a species-specific *L. pneumophila* monoclonal antibody and no reactivity with antibodies to *Legionella bozemanii* serogroups 1 and 2, *Legionella dumoffii* serogroups 1 and 2, *Legionella feeleii*, *Legionella gormanii* serogroup 1, *Legionella jordanii*, *Legionella*

longbeachae serogroups 1 and 2, *Legionella micdadei* serogroups 1 and 2, *Legionella oakridgensis*, *Legionella rubrilucens* and *Legionella sainthelensi*. Immunoblot staining for the presence of *L. pneumophila* IcmX and DotA showed no reactivity with either isolate.

The cellular fatty acids of strain IMVS-3376^T that comprised more than 1% of the identified fatty acids were iso14:0 (3.9%), 14:0 (2.1%), anteiso15:0 (20.4%), 15:1 ω 6c (2.0%), 15:0 (3.9%), iso16:0 (9.0%), 16:1 ω 7c and 16:1 ω 6c (combined 20.1%), 16:0 (23.9%), anteiso17:0 (3.9%), cyclopropane 17:0 (2.6%), 17:0 (2.0%) and 18:0 (1.5%). A repeat analysis from independently grown bacteria gave close results with 10% or less variation from the initial analysis. No *Legionella* species in the MIDI database had matching cellular fatty acid compositions; the closest matches were *L. feeleii*, *L. steigerwaltii*, *L. birminghamensis* and *L. adelaidensis*, with MIDI similarity indices of 0.41, 0.35, 0.28 and 0.26, respectively. MIDI similarity indices of 1.0 and 0.4 indicate complete identity and species level relatedness, respectively.

Both isolates had identical restricted growth temperatures in comparison with *L. pneumophila*, with minimal and very delayed (5–7 days) growth at 37 °C and no growth at 42 °C, on BCYE α medium, with or without 5% CO₂ incubation. In contrast, the bacteria grew well on BCYE α medium at 35 °C, with heavy growth after three days, with a very slight growth enhancement with 5% CO₂ supplementation. Growth at 30 °C was as fast as at 35 °C, though with slightly smaller colonies. There was a one- to two-day growth delay at 25 °C in comparison with growth at 30 or 35 °C. Growth of both isolates in BYE α broth medium was minimal at 35 °C, with no growth above or below that temperature. BCYE α broth supported significantly better growth of both isolates at 35 °C than found for BYE α broth, but no growth occurred at the other temperatures tested.

Intracellular growth in amoebae and human cells.

Isolates IVMS-3376^T and F2632 both grew equally well in cultures of *Acanthamoeba castellanii* at 30 °C, with a 3 log₁₀ increase in bacterial concentrations over a four day period (Fig. 1). In contrast, intra-amoebal growth was less robust at 35 °C, with an ~2 log₁₀ increase in bacterial concentrations over the same time interval. The enhanced growth at 30 °C was the opposite of that observed for the F889 virulent strain of *L. pneumophila*, which grew better in amoebae at 35 °C than at 30 °C.

In contrast to the good growth of strains IMVS-3376^T and F2632 in amoebae at 30 °C, these bacteria failed to multiply, but were not killed, in the human respiratory tract cell line A549, at 35 and 37 °C (Fig. 2). The virulent control strain, *L. pneumophila* F889, grew well at both temperatures, while the avirulent *dotA* mutant strain, F2352, was killed by the A549 cells.

Genotypic characterization. Sequence was recovered from both strains (IVMS-3376^T and F2632) from the following

Fig. 1. Growth of legionella bacteria in *Acanthamoeba castellanii* at 30 and 35 °C. Axes show bacterial concentration versus days of incubation. Not shown for graphical clarity are the data for bacterial strain F2632, which grew almost identically to strain IMVS-3376^T in the amoebae. Results are from triplicate culture wells. Graph represents data from two different experiments. 889, *L. pneumophila* strain F889; 3376, strain IMVS-3376^T.

gene targets: *mip* (614 nt), 16S rRNA (1362 nt), *rpoB* (330 nt), *rnpB* (309 nt) and *proA* (517 nt). For each gene target, identical sequence was recovered from the two strains, confirming their close genetic similarity despite their diverse origins. The phylogenetic analysis determined that the sequences were not congruent with respect to the inferred evolutionary relatedness of individual species, so the gene sequences were analysed separately. The maximum-likelihood analysis of the *mip* gene sequences is presented in Fig. 3. Only the clade containing the

Fig. 2. Growth of legionella bacteria in A549 cells at 35 and 37 °C. Axes show bacterial concentration versus days of incubation. Not shown for graphical clarity are the data for bacterial isolate F2632, which grew almost identically to strain IMVS-3376^T in the cells. Results are from triplicate culture wells. Graph represents data from two different experiments. 3376-37 and 3376-35, isolate IMVS-3376^T at 37 and 35 °C, respectively; 2352-35, *L. pneumophila* dotA mutant F2352 at 35 °C; 889-37 and 889-35, *L. pneumophila* strain F889 at 37 and 35 °C, respectively.

Fig. 3. A maximum-likelihood phylogenetic dendrogram of *mip* sequences from members of the genus *Legionella* that were genetically closest to *L. steelei* sp. nov. All *Legionella* species were included in the analysis, using a GTR + I + G evolutionary model. *Coxiella burnetii* was used as an outgroup. Bootstrap values (1000 replicates) $\geq 70\%$ are displayed. The complete dendrogram is presented in Figure S1. The strain and GenBank accession numbers are included for each *Legionella* species.

genetically most related sister species is presented, although all the specified species were included in the analysis. The complete dendrogram is presented in Fig. S1 (available in IJSEM Online) and a listing of all the species tested is given in the figure legend.

The similarity of the novel strains to the nearest sister species, compared with that between the closest species pair, for each gene target, is presented in Table 1. Note that for each genetic target, the novel species is less similar to other *Legionella* species than the most closely related species pair. The lack of congruency of the inferred evolutionary relationships between species from each genetic target suggests complex evolutionary processes such as recombination events have also occurred. To accommodate this evolutionary model, a SplitsTree network analysis was performed on the concatenated sequences (2973 nt). This analysis confirmed that the novel species was located in a clade that comprises *L. anisa*, *L. bozemaniae*, *L. cherrii*, *L. dumoffii*, *L. gormanii*, *L. parisiensis*, *L. steigerwaltii*, *L. tucsonensis* and *L. wadsworthii* (Fig. S2).

Discussion

Based on their clear genotypic differences from previously described species of the genus *Legionella*, strains IVMS-3376^T and F2632 are proposed as representing a novel species, *Legionella steelei* sp. nov. For each of the five genes studied, the corresponding sequences for the strains of *L. steelei* sp. nov. were distinct from all other species of the

Table 1. Sequence similarity data for *mip*, 16S rRNA, *rpoB*, *rnpB* and *proA* gene sequences

Values in parentheses are percentage nucleotide similarity for either one or both of the shown species.

Gene	Closest sister species to <i>L. steelei</i> sp. nov.*	Most closely related <i>Legionella</i> species†
<i>mip</i>	<i>L. cherrii</i> and <i>L. steigerwaltii</i> (93.6)	<i>L. rubrilucens</i> and <i>L. taurinensis</i> (96.3)
16S rRNA	<i>L. bozemanai</i> and <i>L. steigerwaltii</i> (98.5)	<i>L. erythra</i> and <i>L. rubrilucens</i> (99.3)
<i>rpoB</i>	<i>L. steigerwaltii</i> (92.2)	<i>L. rubrilucens</i> and <i>L. taurinensis</i> (93.6)
<i>rnpB</i>	<i>L. dumoffii</i> (97.4)	<i>L. santacrucis</i> and <i>L. cincinnatiensis</i> (98.7)
<i>proA</i>	<i>L. cherrii</i> (88.0)	<i>L. longbeachae</i> and <i>L. sainthelensi</i> (96.2)

*The most closely related species to *L. steelei* sp. nov. for each gene target.

†The most closely related *Legionella* species pair for each gene target.

genus on the basis of a maximum-likelihood phylogenetic analysis. Further, for every target, the sequence uniqueness was supported by a maximum-likelihood bootstrap analysis (1000 replicates). Such computationally hungry analyses have only recently been made possible by a rapid maximum-likelihood algorithm RAXML and web-based access to large computational resources (Miller *et al.*, 2010). Although the nearest sister species varied for some of the targets, the genetic similarity of the novel strains to the nearest species was greater than that between some other recognized species for each target (Table 1). The SplitsTree analysis (Fig. S2), which is based on a concatenation of the sequences from the five gene targets, confirms the genetic uniqueness of *L. steelei* sp. nov. within a tight cluster that contains *L. anisa*, *L. bozemanai*, *L. cherrii*, *L. dumoffii*, *L. gormanii*, *L. parisiensis*, *L. steigerwaltii*, *L. tucsonensis* and *L. wadsworthii*. With the exception of *L. wadsworthii*, all of species exhibit blue–white fluorescence, as does strain IMVS-3376^T.

This novel species is temperature growth restricted *in vitro*, with minimal growth *in vitro* at temperatures greater than 35 °C, and negligible growth *in vivo* in human cells at either 35 or 37 °C. It has a strong ability to grow well in amoebae at 30 and 35 °C, with superior growth at 30 °C. In contrast, *L. pneumophila* grows well on artificial media over a much broader temperature range, from 25 to 42 °C, and is fully virulent for human cells at 37 °C. The excellent growth of *L. steelei* sp. nov. in amoebae at 30 °C, and its inability to establish a productive infection in human cells suggests that this bacterium is primarily an amoebal pathogen, with little ability to cause disease in humans. Whether either of the patients from whom this bacterium was isolated had Legionnaires' disease is uncertain, but it is unlikely due to the clinical course of the disease and the consultants' opinions. These bacteria were probably isolated from humans because they were transient colonizers, or perhaps contaminants. It is however possible that this bacterium can cause infection in severely immunocompromised patients.

Specific laboratory identification of *L. steelei* sp. nov. using simple phenotypic methods is not possible, although an inability to grow well, or at all, at 37 °C should be a clue to its identity. In addition, production of large amounts of

diffusible bright yellow fluorescent pigment when the isolate is grown on BCYE α medium, particularly if accompanied by cell-bound blue–white autofluorescence would be an additional clue to its identity. Cellular fatty acid analysis yields an apparently unique pattern and such an analysis could be useful for full species identification. Sequencing of the *mip* gene using a standardized method and published databases is the easiest and most specific means of species identification. Use of partial 16S rRNA gene sequencing may, in contrast, lead to incorrect species assignment.

Although not an apparent human pathogen, *L. steelei* sp. nov. may be a valuable model organism to study legionella pathogenesis in low temperature hosts, such as *Dictyostelium discoideum* and zebrafish, assuming that it is virulent at 20 to 25 °C in these hosts. It will also be important to determine whether *L. steelei* sp. nov. has a *dot/icm*-like type IV secretion system, as the failure to detect immunoreactive *L. pneumophila* DotA or IcmX in this study does not exclude such a possibility.

It is possible that *L. steelei* sp. nov. is commonly found in water because it grows well in amoebae at lower temperatures. It may be that its restricted growth temperature has hindered isolation from environmental specimens.

Description of *Legionella steelei* sp. nov.

Legionella steelei (steel'e.i. N.L. masc. gen. n. *steelei* of Steele, pertaining to the Australian microbiologist Trevor Steele, who performed pioneering work on the ecology and pathogenesis of *L. longbeachae* infection).

Gram-negative-staining rod. Grows on BCYE α agar, but not on tryptic soy blood agar or BCYE α agar without L-cysteine. Optimal growth temperature is 35 °C, with no or very poor growth at ≥ 37 °C. Produces large amounts of diffusible yellow–green fluorescent pigment. Variable production of colony-bound blue–white fluorescence. Positive in tests for activities oxidase, catalase, gelatinase, hippurate hydrolysis and β -lactamase. Negative result for glucose utilization. Motile, with monopolar flagella. Dominant cellular fatty acids are anteiso15:0, 16:1 and 16:0. Virulent in *Acanthamoeba*

castellani. 16S rRNA, *mip*, *rpoB*, *rnpB* and *proA* gene sequences differ significantly from all recognized species of the genus *Legionella*.

The type strain, IMVS-3376^T (=IMVS 3113^T=ATCC BAA-2169^T), was isolated from a human respiratory tract specimen.

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