Phylogeny of *Legionellaceae* Based on Small-Subunit Ribosomal DNA Sequences and Proposal of *Legionella lytica* comb. nov. for *Legionella*-Like Amoebal Pathogens

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The 16S rRNA-encoding gene sequences from strains of the family *Legionellaceae*, *Sarcobium lyticum*, and *Coxiella burnetii* were determined. Phylogenetic relationships revealed that all *Legionella* spp. were members of a coherent monophyletic family. The blue-white autofluorescent species formed a defined cluster bounded by *Legionella bozemanii* and *Legionella tucsonensis*. The strains of *Legionella pneumophila* subsp. *pneumophila* and *Legionella pneumophila* subsp. *fraseri* shared 99.2% sequence identity. A legionella-like amoebal pathogen (LLAP-3) showed 99.4% sequence identity to the obligate intracellular bacterial parasite *Sarcobium lyticum*. A proposal is made for the transfer of *Sarcobium lyticum* from the genus *Sarcobium* to the genus *Legionella* as *Legionella lytica* comb. nov. On the basis of serology and phenetic and phylogenetic comparisons, the taxa *Legionella erythra* and *Legionella rubrilucens* may be regarded as subspecies.

The family *Legionellaceae* was initially proposed for a single genus (*Legionella*) and species (*Legionella pneumophila*) (10). Currently, there are 39 validly described species that have been isolated from either clinical or environmental sources or both (28). All species so far described are gram-negative rods, 0.3×10^{-6} to 0.9×10^{-6} m in width and 2×10^{-6} to 20×10^{-6} m or more in length, and polar flagella may be present (40). The legionellae all shared additional phenotypic characters that included absence of growth on blood agar, nitrate not being reduced, negative reaction for urease, a nonfermentative metabolism, a requirement for L-cysteine and iron salts for primary isolation on solid media, respiratory ubiquinones with 10 or more isoprene units, and a predominance of branched-chain cellular fatty acids (5, 6, 34, 52). The moles percent guanine-plus-cytosine content (mol% G+C) ranges from 36.3 (*Legionella cincinnatiensis* [49]) to 52.0 for the type strains of *Legionella geestiana* (16) and *Legionella rubrilucens* (9).

Though bacterial species were defined on the basis of DNA-DNA hybridization data, the interpretation of low DNA hybridization values for some members of the genus *Legionella* resulted in a proposal to divide the family *Legionellaceae* into three genera: *Legionella sensu stricto*, *Tatlockia*, and *Fluoribacter* (12, 26).

In the current study, comparative 16S rRNA-encoding gene (rDNA) sequencing analysis was employed to further delineate and refine the phylogeny of the *Legionellaceae*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA preparation. Strains of *Legionella* (Table 1) were cultured on BCYE agar (20) at 35°C in a humid chamber for up to 7 days. Chromosomal DNA was extracted after lysis with guanidium isothiocyanate reagent (42).

PCR amplification, purification, and automated PCR-directed cycle sequencing of 16S rDNA. Amplification was carried out as previously described (32), and the ca. 1,450-bp product was purified according to the method of Zhen and

Swank (55). PCR products were sequenced directly with the *Taq* DyeDeoxy Terminator kit (Applied BioSystems) according to the manufacturer's protocol. The primers used were pA (8 to 20), pC (= pEM, 341 to 361), pD (518 to 536) and pD⁻ (536 to 518), pH⁺ (1542 to 1522 [35, 36]), pE4 (1113 to 1130 [32]), and 16S1425r (19). The sequence reaction mixtures were electrophoresed with the Applied BioSystems 373A DNA sequencer.

Data analysis. Sequences were aligned against highly conserved regions by the Multalin alignment computer program (15). Regions of extreme hypervariability or ambiguity were removed from the alignment. Phylogenetic analyses were carried out on 46 strains for 1,345 nucleotides with the PHYLIP 3.52c Phylogenetic Inference Package (J. Felsenstein, University of Washington) supported on a SunOS 5.4 workstation at the Human Genome Mapping Program Resource Centre (Cambridge, United Kingdom). The reliability of three nodes was assessed by analyzing multiple data sets ($\times 100$). Unrooted consensus trees were calculated from both parsimony (DNAPARS) and distance methods. Pairwise distances between all sequences were inferred from the Jukes and Cantor (33) one-parameter model (all substitutions are assumed to be equally likely) and under the maximum-likelihood (DNAML) criteria, whereby each branch of the phylogenetic tree was tested for statistical significance. Trees were constructed by neighbor joining (NEIGHBOR [41]) and by using the algorithm of Fitch and Margolish (FITCH [21]). A majority-rule consensus tree was computed from trees derived from multiple data sets ($\times 100$) by the CONSENSE program.

Nucleotide sequence accession numbers. The accession numbers of the *Legionella* nucleotide sequences obtained in this study by automated PCR cycling were as follows: *Legionella adelaidensis*, Z49716; *Legionella anisa*, Z32635; *Legionella birminghamensis*, Z49717; *Legionella bozemanii*, Z49718 and Z49719; *Legionella brunensis*, Z32636; *Legionella cherrii*, Z49720; *L. cincinnatiensis*, Z49721; *Legionella dumoffii*, Z32637; *Legionella fairfieldensis*, Z49722; *Legionella feeleei*, Z49740; *Legionella gormanii*, Z32639; *L. geestiana*, Z49723; *Legionella gratiana*, Z49725; *Legionella israelensis*, Z32640; *Legionella jamestowniensis*, Z49726; *Legionella jordanis*, Z32667; *Legionella lansingensis*, Z49727; *Legionella londiniensis*, Z49728; *Legionella macheachernii*, Z32641; *Legionella moravica*, Z49729; *Legionella nautarum*, Z49730; *Legionella oakridgensis*, Z32643; *Legionella parisiensis*, Z49731; *Legionella quateirensis*, Z49732; *Legionella quinlivanii*, Z49733; *L. rubrilucens*, Z32643; *Legionella sainthelensi*, Z49734; *Legionella santicrucis*, Z49735; *Legionella shakespearei*, Z49736; *Legionella steigerwaltii*, Z49737; *Legionella tucsonensis*, Z32644; *Legionella wadsworthii*, Z49738; *Legionella worsliensis*, Z49739; and the isolate "Glasgow" 86/35785, Z49724. Sequences used for comparison in this study and those obtained by the reverse transcriptase method were as follows: *L. bozemanii*, M36031; *Legionella erythra*, M36027; *Legionella hackeliae*, M36028; *Legionella longbeachae*, M36029; *Legionella micdadei*, M36032; *Legionella pneumophila* subsp. *pneumophila*, M36023; *Legionella pneumophila* subsp. *fraseri*, M36025; *Legionella spiritensis*, M36030; and the isolate LLAP-3, Z49741 (19, 25).

RESULTS AND DISCUSSION

The phenetic relationships among *Legionella* spp. are summarized in Table 1. As previously noted, the genus *Legionella* formed a coherent phenotypic taxon (28), and yet comparisons

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TABLE 1. Phenotypic relationships among *Legionella* species^a

Taxon (EMBL accession no.)	Growth on:		Flagella	Autofluorescence (365-nm UV light)	Bromocresol purple	Growth on		Gelatinase production	Hippuric acid hydrolysis	β -Lactamase production	Oxidase activity	Cellular fatty acids ^b	Isoprenoid quinone ^c	G+C content (mol%) ^d
	BCYE	BCYE-1-cysteine				tyrosine-supplemented agar	Catalase activity							
<i>L. gatlana</i> NCTC 12388 (Z49725 ^e)	+	-	+	-	ND	-	+	+	-	+	±	n-C _{16:1}	Q-10	ND
<i>L. bozemanii</i> WIGA sep 1 NCTC 11368 (M36031 ^f), Mi-15 sep 1 NCTC 11369 (Z49719 ^g), sep 2 NCTC 11976 (Z49718 ^g)	+	-	+	+	+	+	+	+	-	±	±	a-C _{15:0}	ND	ND
<i>L. steigerwaltii</i> NCTC 11991 (Z49737 ^g)	+	-	+	+	-	No growth	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	40
<i>L. wadsworthii</i> NCTC 11532 (Z49738 ^g)	+	-	+	-	-	-	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	ND
<i>L. chertii</i> NCTC 11976 (Z49720 ^g)	+	-	+	+	-	-	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	40
<i>L. gormanii</i> NCTC 11401 (Z32639 ^g)	+	-	+	+	-	-	+	+	-	+	-	a-C _{15:0}	Q-12	ND
<i>L. pneumophila</i> NCTC 11983 (Z49731 ^g)	+	-	+	+	ND	-	+	+	-	+	+	a-C _{15:0}	Q-10-Q-13	42
<i>L. anisae</i> NCTC 11974 (Z32635 ^g)	+	-	+	+	+	+	+	+	-	+	-	a-C _{15:0}	Q-10, Q-12	42
<i>L. dumoffii</i> NCTC 11370 (Z32637 ^g)	+	-	+	+	-	ND	+	+	-	±	-	a-C _{15:0}	Q-12	ND
<i>L. lucosensis</i> NCTC 124439 (Z32644 ^g)	+	-	+	+	+	ND	+	+	-	+	+	a-C _{15:0}	Q-11, Q-12	44
<i>L. samitensis</i> NCTC 11988 (Z49734 ^g)	+	-	+	-	ND	+	+	+	-	ND	-	i-C _{16:0}	ND	ND
<i>L. cinchonensis</i> NCTC 12438 (Z49721 ^g)	+	-	+	+	ND	+	+	+	-	+	+	i-C _{16:0}	Q-10-Q-13	36.3
<i>L. sanitocrus</i> NCTC 11999 (Z49735 ^g)	+	-	+	+	ND	+	+	+	-	+	+	i-C _{16:0}	Q-10-Q-13	38
<i>L. longbeachae</i> NCTC 11477 (M36029 ^g)	+	-	+	+	-	+	+	+	-	+	±	i-C _{16:0}	Q-12	ND
<i>L. pneumophila</i> subsp. <i>pneumophila</i> NCTC 11192 (M36023 ^g), subsp. <i>fraseri</i> NCTC 11233 (M36025 ^g)	+	-	+	-	-	+	+	+	+	+	±	i-C _{16:0}	Q-12	39
<i>L. moravica</i> NCTC 12239 (Z49729 ^g)	+	-	+	-	ND	±	+	+	-	+	±	n-C _{16:1}	ND	41.5
<i>L. quateensis</i> NCTC 12370 (Z49732 ^g)	+	-	+	-	ND	±	+	+	-	+	±	n-C _{16:1}	Q-13	39
<i>L. shakopeae</i> NCTC 12829 (Z49736 ^g)	+	-	+	-	ND	-	+	+	-	+	±	i-C _{16:0}	Q-12	45.5
<i>L. worliensis</i> NCTC 13277 (Z49739 ^g)	+	-	+	-	ND	+	+	+	-	+	+	n-C _{16:1}	Q-13	41
<i>L. feelei</i> NCTC 11978 (Z49740 ^g)	+	±	+	-	-	+	+	+	±	-	-	a-C _{15:0}	Q-13	ND
<i>L. spiritensis</i> NCTC 11990 (M36030 ^g)	+	±	+	-	-	+	+	+	±	+	+	a-C _{15:0}	Q-10-Q-13	46
<i>L. iontiniensis</i> NCTC 12374 (Z49728 ^g)	+	±	+	-	-	+	±	+	±	+	-	a-C _{15:0}	Q-11, Q-13	43
<i>L. brunensis</i> NCTC 12240 (Z32636 ^g)	+	-	+	-	ND	±	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	40.5
<i>L. jordanii</i> NCTC 11533 (Z32667 ^g)	+	-	+	-	-	+	+	+	-	+	-	a-C _{15:0}	Q-13	ND
<i>L. hackeliae</i> NCTC 11979 (M36028 ^g)	+	-	+	-	-	+	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	42
<i>L. jamesoniensis</i> NCTC 11981 (Z49726 ^g)	+	-	+	-	-	+	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	51
<i>L. erythra</i> NCTC 11977 (M36027 ^g)	+	-	+	-	-	+	+	+	-	+	-	i-C _{16:0}	Q-12	52
<i>L. rubilucens</i> NCTC 11987 (Z32643 ^g)	+	-	+	+	+	+	+	+	-	+	-	i-C _{16:0}	Q-12	43
<i>L. quihuvonii</i> NCTC 12433 (Z49733 ^g)	+	-	+	+	ND	+	+	+	-	+	-	a-C _{15:0}	Q-13	42.9
<i>L. birninghamensis</i> NCTC 12437 (Z49717 ^g)	+	-	+	+	ND	-	+	+	-	+	-	a-C _{15:0}	Q-13	42.6
<i>L. lansingensis</i> NCTC 12830 (Z49722 ^g)	+	-	+	+	ND	+	+	+	-	+	-	a-C _{15:0}	Q-13, Q-14	52
<i>L. gestiana</i> NCTC 12373 (Z49723 ^g)	+	-	+	+	ND	+	+	+	-	+	-	a-C _{15:0}	Q-13, Q-14	42
<i>L. fairfieldensis</i> NCTC 12488 (Z49722 ^g)	+	-	+	+	ND	+	+	+	-	+	-	a-C _{15:0}	Q-10-Q-13	43
<i>L. machuchensis</i> NCTC 11982 (Z32641 ^g)	+	-	+	+	±	-	+	+	-	+	-	a-C _{15:0}	Q-13	ND
<i>L. micdadei</i> TATLOCK NCTC 11371 (M36032 ^g)	+	-	+	+	+	-	+	+	-	+	-	a-C _{15:0}	Q-11-Q-13	41
<i>L. israelensis</i> NCTC 12010 (Z32640 ^g)	+	-	+	+	-	-	+	+	-	+	-	i-C _{16:0}	Q-10	43.2
<i>L. oakridgensis</i> NCTC 11531 (Z32643 ^g)	+	-	+	+	-	+	+	+	-	+	-	n-C _{16:1}	Q-11, Q-13	41
<i>L. natronum</i> NCTC 12375 (Z49730 ^g)	+	-	+	+	-	+	+	+	-	+	-	n-C _{16:1}	Q-11	40
<i>L. adalaidensis</i> NCTC 12735 (Z49716 ^g)	+	-	+	+	ND	-	+	+	-	+	-	n-C _{16:1}	Q-11	40

^a Data compiled from references 1 to 4, 8, 13 to 15, 18, 27, 29, 30, 37, 38, and 47 to 54. Symbols and abbreviations: +, positive; -, negative; ±, weak reaction; ND, not done; BW, blue-white autofluorescence; R, red autofluorescence.

^b Predominant constituent(s). i, isobranched; a, anteisobranched; n, normal (straight chain); h, monohydroxy fatty acid. The number following the colon indicates the number of double bonds.

^c Predominant constituent(s). Q, ubiquinones; Q-n, side chain of n isoprenyl units.

^d Determined spectrophotometrically by thermal denaturation.

^e Sequenced by automated PCR cycling (this study).

^f Species implicated in respiratory disease by isolation of the bacterium.

^g Sequenced by reverse transcriptase (19, 25).

FIG. 1. Unrooted consensus maximum likelihood model and Fitch and Margoliash (21) distance tree showing the position of members of the *Legionellaceae*, *S. lyticum*, and *C. burnetii*. Bootstrap percentages quoted are the percentage of times that a taxon occurred at that node. Only values greater than 50% are shown. The scale bar represented 0.02 substitutions per sequence position (K_{nuc}).

of DNA-DNA pairings showed that it was genetically divergent at the species level (5).

All sequences have been submitted to the European Molecular Biology Laboratory, and the accession numbers are given in Table 1. Those for LLAP-3, *Sarcobium lyticum*, "Glasgow" 86/35785, and *Coxiella burnetii* (Fig. 1) were Z49741, X66835, Z49724, and M21291, respectively. The results of the current analysis for 45 legionellae are given in Fig. 1. The phylogenetic tree was outgrouped to the etiological agent of Q fever, *C. burnetii* (Fig. 1) (25).

A comparison of phylogenies based on parsimony, different distance models, and bootstrapped data indicated that the branching order was reliable (Fig. 1). The relative topological positions among *L. bozemanii*, *L. erythra*, *L. hackeliae*, *L. longbeachae*, *L. pneumophila*, *L. micdadei*, and *L. spiritensis* were consistent with the picture provided by Fry et al. (25).

The rate of nucleotide substitution (K_{nuc}) among those le-

gionellae examined varied from 0.0989 (90.7%) for *L. santacrucis* and *L. jamestowniensis* to 0.0053 (99.4%) for the red autofluorescent taxa *L. erythra* and *L. rubrilucens*. Sequence values of between 95 and 97% are generally expected for different species within a genus (46).

The blue-white autofluorescent species (Table 1) formed a well-defined clade bounded by *L. bozemanii* WIGA and *L. tucsonensis* (K_{nuc} value of 0.0218; 97.8%). *L. wadsworthii*, although nonfluorescent, was part of this clade. It is likely that *L. wadsworthii* represented a line of descent to/from a common blue-white autofluorescent ancestor that had lost the ability to synthesize soluble fluorescent pigments. Few differentiating characters are available for this group, but absence of growth on tyrosine-supplemented agar separated *L. steigerwaltii* from others (Table 1).

The serogroup 1 strain of *L. bozemanii* Mi-15 (NCTC 11369) had a higher degree of sequence similarity (99.45% and K_{nuc} of

0.0053) to the type strain of serogroup 2 (NCTC 11975) than to the serogroup 1 reference strain, WIGA (98.7%; K_{nuc} , 0.0128 (Fig. 1). This result may represent some sequence ambiguity inherent within the reverse transcriptase method (25).

Strains of *L. saintheni*, *L. cincinnatiensis*, *L. santicrucis*, and *L. longbeachae* shared a 16S rDNA sequence value of approximately 97.6%, an average K_{nuc} value of 0.0239, and a relatively low mol% G+C of about 39.4 (Table 1).

The two strains of *L. pneumophila* examined and representing different subspecies were recovered in a separated branch. The two *L. pneumophila* subspecies *pneumophila* and *fraseri* exhibited 99.2% sequence identity (K_{nuc} , 0.0079) and could be identified by their ability to hydrolyze hippurate (Table 1). The designation subspecies rather than genospecies (DNA homology values slightly below the 70% cutoff criterion for species designation and indistinguishable phenotype) was, in this instance, the exception to the rule (7).

The isolate LLAP-3 was recovered from the sputum of a patient with pneumonia by cocultivation with *Acanthamoeba polyphaga* (24). On the basis of this study, LLAP-3 showed 99.4% nucleotide identity (K_{nuc} , 0.0060) with another obligate intracellular bacterial parasite, *S. lyticum* (45). There were six nucleotide differences that possibly represented two transitions (both G-A at positions 264 and 473 [*Escherichia coli* nucleotide numbering (11)] and four transversions (two G-C base-pair substitutions and A-T and C-A at positions 664, 1218, 261, and 1124, respectively). Both strains resided within the *Legionellaceae*, and the nearest, yet unrelated, species was *L. pneumophila*. Since the genus *Legionella* (originally described in 1979 by Brenner et al. [10]) has precedent over *Sarcobium* (*S. lyticum* [17]) according to the Rule of Nomenclatural Priority (44), a proposal is made for the transfer of the species *S. lyticum* from the genus *Sarcobium* to *Legionella* as *Legionella lytica* (17) comb. nov. The description of *L. lytica* comb. nov. is the same as that published for *S. lyticum* (17). Cells were gram negative and motile by means of three to five polar flagellar rods that were 0.6×10^{-6} to 1.9×10^{-6} m. The DNA base composition as determined by thermal denaturation was 43 mol% guanine plus cytosine. The organism has chemotype A_{1y} peptidoglycan with a free amino group on the glucosamine residues. It is an obligate intracellular parasite of amoebae that belong to the *Acanthamoeba-Naegleria* group of protozoa. The type strain was deposited with the Polish Culture Collection as culture PCM 2298. A similar strain (99.4% 16S rDNA similarity [this study]) would be LLAP-3 (24).

The environmental isolates *L. moravica*, *L. quateirensis*, *L. shakespearei*, and *L. worshiensis* had straight-chain or iso-branched cellular fatty acids (n-C_{16:1}, i-C_{16:0}), an approximate mol% G+C content of 41.7 (Table 1), and an average K_{nuc} value of 0.0254 (97.45%).

The clinical isolate "Glasgow" 86/35784 had unusual colonial yellow autofluorescence under UV light, a nucleotide substitution rate with a K_{nuc} of 0.0294, and low similarity (94.1%) to its nearest relative, *L. feelei*. At this level of dissimilarity, it is likely that this strain does represent a new and novel species within the family *Legionellaceae*.

The legionellae *L. spiritensis*, *L. londiniensis*, *L. brunensis*, *L. jordanis*, *L. hackeliae*, and *L. jamestowniensis* formed a relatively large segment of the inferred phylogenetic tree. The moles percent G+C varied from 40 for *L. hackeliae* to 46 for *L. spiritensis*, distance values were from 0.0642 in K_{nuc} (93.8%; *L. spiritensis*-*L. jamestowniensis*) to 0.0326 in K_{nuc} (96.8%; *L. londiniensis*-*L. brunensis*), and they had a similarity value of about 95.2%. The phenotypes of these species were not dissimilar, and yet *L. spiritensis* and *L. londiniensis* exhibited vari-

able growth on BCYE without L-cysteine supplement and variation in their ability to hydrolyze hippurate (Table 1).

The red autofluorescent taxa *L. erythra* and *L. rubrilucens* were described in 1985 (9) on the basis of DNA pairing studies. Given that strains cross-reacted serologically (43) and have similar biochemical and chemotaxonomic states (Table 1), identical intergenic 16S-23S rDNA length polymorphisms (31), comparable sequence comparisons (99.4% 16S rDNA [this study]), and DNA hybridization values just below (43) the 70% cutoff level for species designation, these taxa could be considered subspecies of the same species.

L. quinlivanii, *L. birminghamensis*, *L. lansingensis*, and *L. geestiana* represented a divergent assembly sharing an approximate K_{nuc} value of 0.0621 and exhibiting sequence variation from 97.1% (*L. quinlivanii*-*L. birminghamensis*) to 91.7% (*L. lansingensis*-*L. geestiana*) and mol% G+C from 52.0 (*L. geestiana*) to 42.6 (*L. lansingensis*).

L. fairfieldensis was loosely related at 0.0509 in K_{nuc} (96.45%) to *L. maceachernii* and *L. micdadei*. The organism was nonautofluorescent, did not degrade gelatin, exhibited variable oxidase activity, and had a mol% G+C of 42 (Table 1; Fig. 1).

Similarities existed between *L. maceachernii* and *L. micdadei*, and yet they differed from other legionellae in giving a positive bromocresol purple spot reaction (26). Garrity and others (12, 26) interpreted low DNA homologies between some strains of legionellae to subdivide the family into three genera, namely, *Legionella* sensu stricto, *Tatlockia* (*maceachernii* and *micdadei* [23]), and *Fluoribacter* (*bozemanii*, *gormanii*, and *dumoffii* [12]). However, high levels of 16S rDNA sequence divergence for some species within a genus are not unknown (22) and should not be used as an argument for splitting a coherent phenotypic family into multiple genera. The results of the current study showed that there are no discrete clusters, as would be expected for a polyphyletic classification. We conclude that all *Legionella* spp. should be regarded as members of a coherent monophyletic family which belong to the gamma subdivision of the *Proteobacteria* (25).

On the periphery of the tree were the legionellae *L. israelensis*, *L. oakridgensis*, *L. nautarum*, and *L. adelaidensis*. They shared K_{nuc} values from 0.0714 to 0.0464 (93.1 to 95.5%). The absence of polar or lateral flagella and growth on BCYE without an L-cysteine supplement after primary isolation, in part, characterized *L. oakridgensis*.

The phylogenetic structure of the *Legionellaceae* based on 16S rDNA sequence comparisons shows concord with the existing phenetic classification and also supports the call for establishing a polyphasic approach to taxonomy (39).

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