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Legionella drozanskii sp. nov., *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov.: three unusual new *Legionella* species

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Seven strains of *Legionella*-like amoebal pathogens (LLAPs) were characterized on the basis of their cultural and staining characteristics, biochemical reactions, serology, cellular fatty acids (CFAs), isoprenoid quinone composition, total DNA relatedness, analysis of 16S rRNA and macrophage infectivity potentiator (*mip*) gene sequence analyses. All seven strains exhibited limited growth on buffered charcoal yeast extract α (BCYE) agar, required cysteine for growth and contained branched-chain CFAs and quinones typical of *Legionella* species. The bacilli were Gram-negative and catalase-positive. There were varying degrees of serological cross-reactions between these LLAP strains and other previously described *Legionella* species. Results from the various tests revealed that four LLAP strains represent three unusual new species of *Legionella*: *Legionella drozanskii* sp. nov., type strain LLAP-1^T; *Legionella rowbothamii* sp. nov., type strain LLAP-6^T; and *Legionella fallonii* sp. nov., type strain LLAP-10^T. Three other LLAP strains, designated LLAP-7FL, LLAP-7NF and LLAP-9, were shown to be members of the species *Legionella lytica.* The deductions made from the phenetic characteristics of these bacteria were consistent with the phylogenetic relationships inferred from 16S rRNA and *mip* gene sequence analyses. This study is the first to speciate LLAP strains on the basis of data including quantitative DNA hybridization.

Keywords: amoeba, autofluorescence, branched-chain fatty acids, *mip*, DNA hybridization

INTRODUCTION

Members of the genus *Legionella* are the established aetiological agents of Legionnaires' disease and its milder relative, Pontiac fever. Legionellae have been described as obligate parasites of single-celled protozoa (Rowbotham, 1986; Fields, 1996). Similarly, *Legionella*-like amoebal pathogens (LLAPs) are primarily obligate intracellular parasites of amoebae (Adeleke *et al.*, 1996). These bacteria derived their name from their ability to infect and multiply in amoebae in an identical way to members of the genus *Legionella* (Rowbotham, 1986; Fields *et al.*, 1990). LLAP-3 was isolated from the sputum of a pneumonia patient, following co-culture of the sputum with amoebae. All the other LLAP strains (except *Legionella lytica*) were originally isolated from sites sampled during investigations into individual cases or clusters of cases of Legionnaires' disease. There is also serological evidence associating LLAPs with respiratory disease in humans (Rowbotham, 1993; Benson *et al.*, 1995).

The grouping together of members of the genus *Legionella* is based on phenotypic similarities and is supported by molecular techniques (Brenner *et al.*,

Abbreviations: BCYE, buffered charcoal yeast extract α; CFA, cellular fatty acid; LLAP, *Legionella*-like amoebal pathogen; SAT, slide agglutination test.

The GenBank accession numbers for approximately 600 bases of sequence of the *mip* gene of *Legionella lytica* and strains LLAP-1^T, LLAP-6^T, LLAP-7, LLAP-9, LLAP-10^T and 4313-GER-E are AF148982, AF148983, AF148984, AF148985, AF148986, AF148987 and AF148988, respectively.

Table 1. Bacterial strains

NYA, Not yet accessioned.

Legionella species/serogroup	Serogroup	ATCC no.	Original designation
L. pneumophila	Sgp1	33152	Philadelphia 1^T
	Sgp2	33154	Togus 1
	Sgp3	33155	Bloomington 2
	Sgp4	33156	Los Angeles
	Sgp5	33216	Dallas 1
	Sgp6	33215	Chicago 2
	Sgp7	33823	Chicago 8
	Sgp8	35096	Concord 3
	Sgp9	35289	IN-23-G1-C2
	Sgp10	43283	Leiden 1
	Sgp11	43130	797-PA-H
	Sgp12	43290	570-CO-H
	Sgp13	43736	82A3105
	Sgp14	43703	1169-MN-H
	Sgp15	35251	Lansing 3
L. adelaidensis		49625	1762-AUS-E^T
L. anisa		35292	WA-316-C3^T
L. birminghamensis		43702	1407-AL-H^T
L. bozemanii	Sgp1	33217	WIGA^T
	Sgp2	35545	Toronto 3
L. brunensis		43878	444-1^T
L. cherrii		35252	ORW^T
L. cincinnatiensis		43753	72-OH-H^T
L. dumoffii		33279	NY-23^T
L. erythra		35303	SE-32-A-C8^T
L. fairfieldensis		49588	1725-AUS-E^T
L. feeleii	Sgp1	35072	WO-44C^T
	Sgp2	35849	691-WI-H
L. geestiana		49504	1308^T
L. gormanii		33297	LS-13^T
L. gratiana		49413	Lyon 8420412^T
L. hackeliae	Sgp1	35250	Lansing 2^T
	Sgp2	35999	798-PA-H
L. israelensis		43119	Bercovier 4^T
L. jamestowniensis		35298	JA-26-G1-E2^T
L. jordanis		33623	BL-540^T
L. lansingensis		49751	1677-MI-H^T
L. londoniensis		49505	1477^T
L. longbeachae	Sgp1	33462	Long Beach 4^T
	Sgp2	33484	Tucker 1
L. lytica		NYA	L2^T
L. maceachernii		35300	PX-1-G2-E2^T
L. micdadei		33218	TATLOCK^T
L. moravica		43877	316-36^T
L. nautarum		49506	1224^T
L. oakridgensis		33761	Oak Ridge 10^T
L. parisiensis		35299	PF-209C-C2^T
L. quateriensis		49507	1335^T
L. quinlivanii	Sgp1	43830	1442-AUS-E^T
	Sgp2	NYA	LC870
L. rubrilucens		35304	WA-270-C2^T
L. sainthelensi	Sgp1	35248	Mt St Helens^T
	Sgp2	49322	1489-CA-H

Table 1 (*cont.*)

Legionella species/serogroup	Serogroup	ATCC no.	Original designation
L. santicrucis		35301	SC-63-C7^T
L. shakespearei		49655	214^T
L. spiritensis	Sgp1	35249	Mt St Helens 9^T
L. steigerwaltii		35302	SC-18-C9^T
L. tucsonensis		49180	1087-AZ-H^T
L. wadsworthii		33877	81-716A^T
L. waltersii		51914	2074-AUS-E^T
L. worsleiensis		49508	1347^T
Legionella genomospecies 1		51913	2055-AUS-E

1985; Brenner, 1986; Fry *et al*., 1991; Harrison & Saunders, 1994; Hookey, 1995; Benson *et al*., 1996; Hookey *et al*., 1996; Ratcliff *et al*., 1997, 1998). There are few discriminatory phenotypic tests for the differentiation of *Legionella* species (Benson & Fields, 1998; Harrison & Saunders, 1994). Hence, definitive speciation relies on a variety of methods, the most conclusive of which are DNA homology studies (Brenner, 1986; Fox *et al*., 1992; Harrison & Saunders, 1994; Benson *et al*., 1996). Recently, rapid identification of legionellae has been significantly enhanced with the development of a genotyping scheme based on the *mip* gene (Ratcliff *et al*., 1998).

Unlike legionellae, LLAPs exhibit little or no growth on bacteriological media and the cultivation of these bacteria on laboratory media cannot be considered routine. Consequently, the characterization and classification of this interesting group of bacteria has been limited. The only LLAP strain that has been validly named was classified in the absence of DNA homology data (Drozanski, 1991; Hookey *et al*., 1996). This strain was initially classified in a separate genus as *Sarcobium lyticum*, based on the phenotypic features of the organism and its obligate intracellular existence or pathogenicity for amoebae (Drozanski, 1991). However, *S. lyticum* was subsequently transferred to the genus *Legionella* as *L. lytica*, on the basis of 16S rRNA sequence analysis (Hookey *et al*., 1996).

The current study was conducted to determine the taxonomic identity of LLAPs. The study adopted a polyphasic approach involving the use of phylogenetic relationships, cultural and staining characteristics, biochemical and serological reactions, as well as chemotaxonomic analyses, including the analysis of cell wall components and total DNA relatedness (Murray *et al*., 1990; Priest & Austin, 1993).

METHODS

Strains. *L. lytica* was provided by Professor W. Drozanski of Maria-Curie-Sklodowska University, Lublin, Poland, while LLAP-1^T, LLAP-6^T, LLAP-7, LLAP-9 and LLAP-10^T were supplied by Dr T. J. Rowbotham of the Public Health Laboratory, Leeds, UK. A previously unidentified *Legionella*-like strain, 4313-GER-E, a drinking water isolate, was supplied by Dr Rolf Michel of the Ernst-Rodenwaldt-Institut, Koblenz, Germany, to CDC for identification.

Type and reference strains representing 40 species, 1 genomospecies and 63 serogroups of *Legionella* were used (Table 1). In addition, 15 unnamed *Legionella*-like organisms (1–15) were also studied.

Isolation. *L. lytica* was isolated as a bacterial infection of *Acanthamoeba*. The strain was found in a culture of a small free-living amoeba isolated from Polish soil in 1954 (Drozanski, 1956; Schlecht & Drozanski, 1987). LLAP-1^T, LLAP-6^T, LLAP-7, LLAP-9 and LLAP-10^T were isolated via amoebal enrichment from various sources in the UK. The strains were isolated between 1981 and 1994 following co-culture of respective samples with amoebae as described by Rowbotham (1983, 1993). The sources of the samples included a tank of potable well water, liquefier tower, whirlpool spa, factory cooling water and a ship air-conditioning system. In some instances, known *Legionella* species were isolated from the same sources. Although each of these sources was associated with confirmed cases of Legionnaires' disease, there were instances when no legionellae were isolated by conventional techniques. For example, LLAP-7 was isolated from a tidemark sample of a whirlpool spa bath in a hotel in Leicestershire, UK in 1991. The spa was associated with two cases of Legionnaires' disease where the patients had serological evidence of infection with *Legionella pneumophila* serogroup 1. *L. pneumophila* serogroup 1 was detected by immunofluorescence in a smear of the tidemark sample while LLAP-7 was isolated following co-culture of the same sample with *Acanthamoeba palestinensis* at 35 °C. *L. pneumophila* serogroup 1 was also isolated from the biofilm in the overflow channel after co-culture with *A. palestinensis* at 35 °C. However, no legionellae were isolated from either sample by conventional culture. A variety of protozoa, including flagellates, ciliates, amoebae, bacteria-infected amoebae and amoebal cysts, were seen in the original samples.

Co-culture of LLAP strains. All LLAP strains, including *L. lytica* were co-cultured with suitable host amoebae at 30 or 35 °C (Adeleke, 1998). Co-culture samples containing accumulations of extracellular bacteria were inoculated onto buffered charcoal yeast extract α (BCYE; Edelstein, 1981) agar plates and incubated at 30 °C. Single colonies from each LLAP strain were subcultured on BCYE and grown at 30 °C.

Growth and biochemical tests. LLAP strains, including *L. lytica*, were grown on BCYE at 30 °C while all *Legionella*

and the unnamed *Legionella*-like strains were grown on BCYE at 35 °C. Gram staining and determination of oxidase and catalase activities, L-cysteine requirement, colony autofluorescence and glucose utilization were carried out as described by Brenner *et al.* (1985).

Cellular fatty acids (CFAs) and isoprenoid quinones. Bacterial cells to be used for fatty acid and quinone analyses were grown on BCYE for 72–96 h at 30 °C. Cellular lipids were saponified and the liberated fatty acids were methylated and analysed by capillary GLC (Weyant *et al.*, 1996) using a commercially available software package (MIDI, Newark, DE, USA). The amide-linked hydroxy acids that were not totally released by this saponification procedure were completely released by a subsequent acid hydrolysis of the methanolic aqueous layer after the methylation step (Weyant *et al.*, 1996). The identification of fatty acids and determination of double bond positions in monounsaturated acids were accomplished by GLC and GLC-MS. The confirmation of hydroxy acids was accomplished by both acetylation and GLC-MS analysis as previously described (Weyant *et al.*, 1996).

Isoprenoid quinones were extracted from 100 mg lyophilized cells and were analysed by reverse-phase HPLC and MS (Moss & Guerrant, 1983).

Serology. Formalin-killed antigen suspensions of LLAP strains were tested with rabbit antisera prepared against the type strains of 40 species and 1 genomospecies, and included 63 serogroups by the slide agglutination test (SAT) (Thacker *et al.*, 1985; Benson *et al.*, 1996).

16S rRNA sequence analysis. Genomic DNA was extracted from LLAP strains and amplified using PCR. The 16S rRNA gene sequences were then determined and analysed as described previously (Adeleke *et al.*, 1996; Birtles *et al.*, 1996).

Genotyping. Genotyping, utilizing the *mip* gene, was performed by gene amplification and dye terminator chemistry (Applied Biosystems) as described by Ratcliff *et al.* (1998). The sequences were analysed and a UPGMA dendrogram produced using the GeneCompar program (Applied Maths).

DNA hybridization. DNA from LLAP-1^T, LLAP-6^T, LLAP-7FL, LLAP-7NF, LLAP-9, LLAP-10^T and *L. lytica* was extracted, purified, enzymically labelled and tested in reassociation experiments against one another, type strains of 36 validly named *Legionella* species, 14 unnamed species and the *Legionella*-like strain 4313-GER-E. *Legionella anisa*, *Legionella rubrilucens*, *Legionella cherii*, *Legionella steigerwaltii*, *Legionella santicrucis* and unnamed strain RI 227 were not included in the tests because each of them share at least 50 % DNA relatedness with a *Legionella* species that was tested. DNA relatedness was determined by the hydroxyapatite method at 60 °C. All experiments were performed at least twice. The methods for DNA extraction, purification and hybridization have been described by Brenner *et al.* (1978).

RESULTS

Cultural characteristics

All seven strains showed limited growth on BCYE and required cysteine for growth. No growth was observed on Trypticase soy agar and blood agar plates. All early grown colonies had a characteristic grey-blue colour and ground glass appearance. However, after prolonged incubation, each strain developed its own distinctive morphology. LLAP-6^T colonies developed a bright yellow pigmentation. Colonies of strain 4313-GER-E were found to have an identical appearance and colour to LLAP-6^T. LLAP-6^T, LLAP-9 and *L. lytica* exhibited blue-white autofluorescence on exposure to long wavelength UV light, while LLAP-1^T and LLAP-10^T did not autofluoresce. When grown on BCYE, two colony types were observed in LLAP-7 cultures. One type showed blue-white autofluorescence while the other was non-fluorescent on exposure to long wavelength UV light. The strains were designated LLAP-7FL and LLAP-7NF, respectively. In natural light LLAP-7FL colonies had an olive to yellow colour. For all strains, cells were Gram-negative and catalase-positive. All strains were oxidase-negative, except for LLAP-10^T which had a weakly positive oxidase reaction. None of the strains produced acid from D-glucose.

CFA and isoprenoid quinone content

The CFA compositions of the strains tested are presented in Table 2. All strains in the current study contained high concentrations (> 20 %) of branched-chain fatty acids. The major or predominant branched-chain fatty acids included a-15:0, i-16:0 and a-17:0 with smaller amounts (0–9 %) of several other branched acids, including i-13:0, a-13:0, i-14:0, i-15:0, i-16:1?, i-17:0, i-18:0, i-19:0 and a-19:0. Straight-chain and other acids detected included 12:0, 14:1?, 14:0, 15:1ω6*c*, 15:0, 16:1ω7*c*, 16:0, 17:0cyc, 17:0, 18:0, 19:0 and 20:0. Small to moderate amounts (trace–11 %) of branched- or straight-chain, ester-linked hydroxy acids were detected in some strains. In the case of LLAP-6^T, LLAP-9 and 4313-GER-E, these ester-linked hydroxy acids were useful for classification and differentiation.

Examination of the qualitative and quantitative fatty acid data showed that the strains tested could be placed into at least four major CFA groups (Table 2).

All the strains tested contained ubiquinones with 10, 11, 12, 13 and 14 isoprene units (Q-10, Q-11, Q-12, Q-13 and Q-14, respectively) in the polyprenoid side chain and no menaquinones were detected. The major ubiquinones in all strains were Q-12 and Q-13, which were present at a concentration approximately four times the concentration of Q-10, Q-11 and Q-14.

Serological reactions

LLAP-1^T showed varying degrees of cross-reaction with other *Legionella* species and serogroups in SATs. LLAP-6^T showed strong (4+) cross-reactions to both unabsorbed and absorbed *Legionella bozemanii* serogroup 2. Strong (4+) reactions were also noted with unabsorbed antisera to *L. bozemanii* serogroup 1 and *Legionella longbeachae* serogroup 2. However, no reactions were observed with the corresponding absorbed antisera. There were notable (4+) reactions to

Table 2. CFA compositions of LLAP strains and strain 4313-GER-E

Values are the percentage of total fatty acids and are given as arithmetic ranges; T, trace (0·4–0·8 %). Numbers before colons refer to the number of carbon atoms; numbers after colons refer to the number of double bonds; OH, hydroxy group at the 2(α) or 3(β) position from the carboxyl end; i, methyl branch at the iso carbon atom; a, methyl branch at the anteiso carbon atom; ω, double bond position from the hydrocarbon end of the carbon chain; *c*, *cis* isomer; cyc, cyclopropane ring structure; ?, position of the double bond is unknown.

Strain	Group 1	Group 2				Group 3	Group 4	
	LLAP-1	LLAP-7FL	LLAP-7NF	LLAP-10	*L. lytica*	LLAP-9	LLAP-6	4313-GER-E
3-OH-10:0	–	–	–	–	–	2	–	–
12:0	–	–	T	–	–	2	–	–
3-OH-12:0	–	–	–	–	–	3	–	–
1-13:0	–	–	–	–	–	–	2–3	3–5
a-13:0	T–1	0–2	0·9–1	–	1–2	1–2	0–3	–
1-14:0	1–3	6–9	4–5	4–5	6–9	2	5	4–5
14:1?	2–3	1	1	1	1–2	1–2	1–2	2–3
14:0	4–6	0–1	1	2–3	2–3	3–6	2–3	3–5
i-15:0	T–1	–	T	T	–	–	5	5–6
a-15:0	27–30	25–42	47–51	12–15	28–29	9–14	12–16	15–17
15:1ω6*c*	5–7	1–2	0–1	3–4	1–2	1	2–3	2–3
15:0	2–3	–	0–T	2–3	2	1	1–2	1–2
3-OH-14:0	–	–	–	–	0–T	2–11	0–T	0–1
i-16:1?	1–2	–	0–T	–	–	–	–	–
i-16:0	9–10	5–10	5–7	11–13	4–6	1–2	4–5	2–5
16:1ω7*c*	18–20	11–15	13–14	21–27	10–14	23–26	16–22	17–27
16:0	6–10	4–8	5–10	15–19	10–14	14–18	9–10	9–12
i-3-OH-15:0	–	–	–	–	–	–	2–4	1–4
2-OH-15:0	–	–	–	–	–	T–3	–	0–1
i-17:0	–	–	–	–	–	–	4–5	2–5
a-17:0	3–6	5–11	7–10	2–3	6–7	2–4	3–4	2–4
17:0cyc	2–6	0–8	2–10	2–6	8–10	2–3	5–8	1–11
17:0	–	1–3	T	2	2	1–2	3	1–2
i-18:0	–	2–8	1	T	2–5	1	2–6	0–2
18:0	–	1–5	1	6–7	3–4	6–10	4	2–4
i-19:0	–	–	–	–	–	–	0–1	0–1
19:0	–	–	–	1–2	–	–	–	–
a-19:0	–	1–4	T	0–T	1–2	1–2	0–1	0–1
20:0	1–4	–	–	1–2	–	–		

unabsorbed *Legionella anisa* serogroup 1, but the corresponding absorbed serum was unavailable. Cross-reactions between LLAP-6^T and antisera to *L. bozemanii* serogroup 2 and to *L. anisa* serogroup 1 were noted in SATs. Strain 4313-GER-E reacted strongly (4+) with unabsorbed *L. longbeachae* serogroup 1 but not with the absorbed antiserum. LLAP-7FL showed significant reactions (3+) to both absorbed and unabsorbed *L. longbeachae* serogroup 1. Like LLAP-7FL, LLAP-7NF showed strong (4+) reactions to absorbed and unabsorbed *L. longbeachae* serogroup 1 in SATs. No further cross-reactions were noted between LLAP-7NF and other *Legionella* strains and serogroups. LLAP-9 showed considerable (4+) reactions to antiserum to *Legionella wadsworthii*. *L. lytica* had significant (3+/4+) reactions to *L. bozemanii* serogroup 2, *L. longbeachae* serogroups 1 and 2 and *Legionella spiritensis* serogroup 1. LLAP-10^T reacted strongly (4+) with both unabsorbed and absorbed sera to *Legionella feeleii* serogroup 2 in SATs.

16SrRNA sequence analysis

The 16S rRNA sequence similarity over 1303 bases between the LLAP strains tested and those of previously described *Legionella* species was 93·4–97·0 %. The results of sequence analyses and GenBank accession numbers for the LLAP strains have been published previously (Adeleke *et al.*, 1996; Birtles *et al.*, 1996).

Genotyping

Approximately 600 bases of sequence of the *mip* gene was determined for each LLAP strain. The sequences

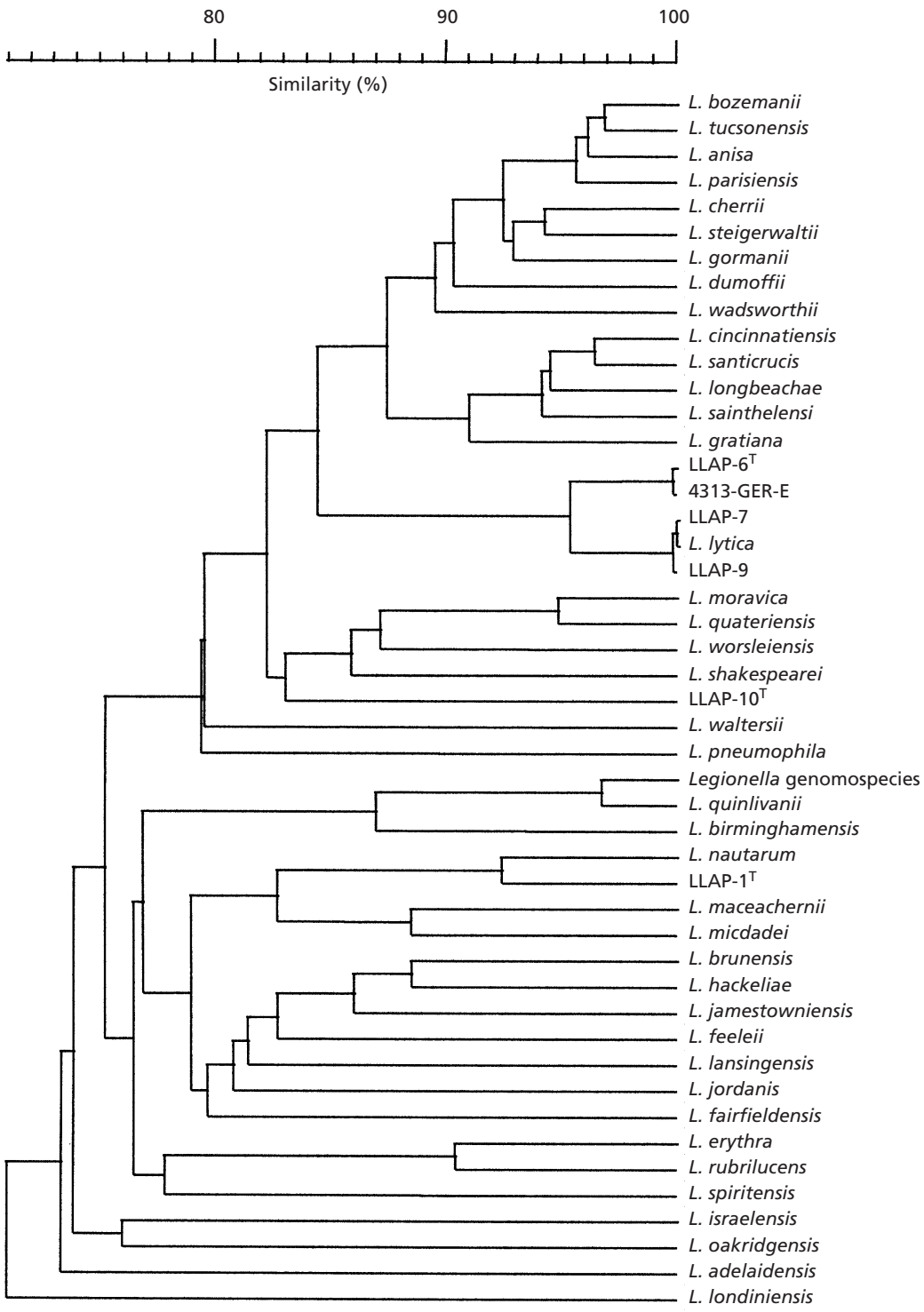

Fig. 1. UPGMA phylogenetic dendrogram of sequence similarities found among *Legionella* species and LLAP strains. The horizontal bar joining two isolates or clusters indicates the level of similarity.

were compared with those published for the *mip* gene from other species (Ratcliff *et al*., 1998). The UPGMA phylogenetic dendrogram of interspecies relatedness is presented in Fig. 1. LLAP-1^T was 92·4% similar to *Legionella nautarum* and less than 84% similar to all other *Legionella* species. LLAP-6^T and 4313-GER-E were 99·8% similar to each other. *L. lytica*, LLAP-7 and LLAP-9 were clustered together with a similarity of 99·8%. The latter two clades were 95% related to each other, demonstrating a degree of relatedness, but

< 85% similar to all other species. LLAP-10^T was < 86% similar to any other species.

DNA homology studies

On the basis of total DNA relatedness as determined by the hydroxyapatite method at 60 °C, LLAP-1^T was 62·5% related to *L. nautarum* but < 24% related to all other strains. LLAP-6^T and 4313-GER-E had a total DNA relatedness of 91%. Similarly, *L. lytica*, LLAP-7NF, LLAP-7FL and LLAP-9 were 92·5% related. *L. lytica* was < 14% related to all other strains (Giles *et al.*, 1995), except for LLAP-6^T to which it was related by 56·5%. LLAP-10^T was < 19% related to all the species tested. The divergence between strains that were related by > 90% was < 1%.

DISCUSSION

The family *Legionellaceae* was originally created for one genus, *Legionella* (Brenner *et al.*, 1984). Consequently, the characteristics of both the family and the genus are essentially the same. Members of the *Legionellaceae* and the genus *Legionella* are Gram-negative, catalase-positive and oxidase-negative or weakly positive (Orrison *et al.*, 1983; Brenner *et al.*, 1985; Verma *et al.*, 1992; Harrison & Saunders, 1994; Benson *et al.*, 1996). Amongst other significant similarities shared by members of the family is the requirement of L-cysteine for growth on artificial medium and an inability to ferment or oxidize carbohydrates (Brenner *et al.*, 1984; Holt *et al.*, 1994). The LLAP strains tested were presumptively identified as legionellae based on these characteristics.

Furthermore, the grey-blue colour and cut glass appearance seen in early growth of all LLAP colonies is typical of most legionellae. The morphology of legionellae may vary under adverse conditions of growth (Wilkinson, 1988; Paszko-Kolva *et al.*, 1992). Therefore, it is not unusual to find that LLAP strains which were newly adapted to growth on bacteriological media exhibited distinct and unusual morphology after prolonged incubation. The ability of some *Legionella* species to autofluoresce when exposed to long wavelength UV light (366 nm) proves useful for differentiating such isolates. For example, *L. rubrilucens* and *Legionella erythra* are known to exhibit red autofluorescence while a number of species, including *L. bozemanii*, *Legionella parisiensis* and *L. anisa* (Brenner *et al.*, 1985) are known to exhibit blue-white autofluorescence. Likewise, LLAP-6^T, LLAP-7FL, LLAP-9, *L. lytica* and 4313-GER-E exhibited blue-white autofluorescence while LLAP-1^T and LLAP-10^T did not autofluoresce.

Members of the genus *Legionella* have been found to differ from other Gram-negative bacteria by the presence of relatively large amounts of branched-chain fatty acids and the absence or presence of trace amounts of hydroxy acids (Lambert & Moss, 1989). Their cell wall usually contains major ubiquinones with ten or more isoprene units in the side chain (Karr *et al.*, 1982). Of the numerous species of bacteria to which they were compared, the overall CFA profiles and quinone contents of the LLAP strains tested were most similar to those of one another and other legionellae. However, comparing the presence of and/or relative amounts of saturated, unsaturated, branched, cyclopropane and hydroxy acids enabled differentiation into four CFA groups. The fatty acid composition of LLAP-1^T differed from those of the other three groups (Table 2) by the presence of i-16:1? (1–2%) and larger amounts (> 5%) of 15:1ω6c and the absence of 17:0, i-18:0 and 18:0. LLAP-7NF, LLAP-7FL and LLAP-10^T (Group 2 strains) differed from strains of other CFA groups by the presence of larger amounts (4–9%) of i-14:0 and the absence of hydroxy acids (except for 0–trace amounts of 3-OH-14:0 in *L. lytica*). LLAP-9 (Group 3) was distinguished from strains of other CFA groups by the presence of 12:0 (2%) and four hydroxy acids: 3-OH-10:0, 3-OH-12:0, 3-OH-14:0 and 2-OH-15:0 (trace–11%). The CFA compositions of LLAP-6^T and 4313-GER-E differed from those of other groups by the presence (1–5%) of i-3-OH-15:0, i-13:0 and i-17:0 as well as larger amounts (5–6% vs trace–1%) of i-15:0. In summary, all of the strains tested contained large amounts of branched-chain acids and in some cases small to moderate amounts of ester-linked hydroxy acids. The presence of such ester-linked hydroxy acids was useful for the classification and differentiation of Groups 3 and 4. In addition, all of the strains tested contained ubiquinones in which the isoprenoid side chain contained ten or more isoprene units.

Despite varying degrees of cross-reactions, it was clear that LLAP-1^T, LLAP-6^T, LLAP-9 and LLAP-10^T were distinct in their serological reactions, while LLAP-7NF, LLAP-7FL and *L. lytica* were similar on the basis of their serological reactions. Further tests may be needed to clarify the serogrouping of these strains.

Based on a DNA reassociation value of 70% and divergence of less than 5% at optimum temperature (60 °C) between members of the same species (Priest & Austin, 1993), DNA reassociation experiments confirmed that LLAP-1^T, LLAP-6^T and LLAP-10^T represent three new species of *Legionella*. On the other hand, LLAP-7NF, LLAP-7FL, LLAP-9 and *L. lytica* belong to the same species. However, LLAP-9 is phenotypically distinct from the other strains of the same species and possibly represents a different serogroup from LLAP-7NF, LLAP-7FL and *L. lytica*.

Based on the level of *mip* gene similarity between the currently described species of *Legionella* (Ratcliff *et al.*, 1998), the level of similarity determined for LLAP-1^T, LLAP-6^T and LLAP-10^T is consistent with each of the three isolates representing a new species. LLAP-1^T showed a degree of relatedness to *L. nautarum* consistent with the total DNA homology findings. Similarly, LLAP-6^T showed a degree of relatedness to *L.*

lytica, also consistent with the total DNA homology findings. *L. lytica* was recognized as a distinct species by this scheme, being less than 14% related to all other published species. LLAP-7 and LLAP-9 exhibit the same genotype as strains of *L. lytica*. Strains 4313-GER-E and LLAP-6^T had a near identical *mip* gene sequence, indicative of belonging to the same species, in spite of the increased culturability of 4313-GER-E on bacteriological media. At least one other strain (Strain TE-1) is known to exist which exhibits the same genotype as LLAP-6^T, but was isolated on BCYE medium after extended incubation at 30 °C in a candle jar (V. Drasar, personal communication). All of these genotyping results parallel the findings and relationships determined from total DNA homology results, indicating that genotyping based on the *mip* gene continues to effectively discriminate newly characterized species when the percentage DNA homology is low.

The phenetic characteristics of the LLAP strains studied, including their growth and cultural characteristics, biochemical reactions, CFA profiles and serological reactions are in agreement with previous inferences from phylogenetic analyses (Adeleke *et al.*, 1996; Birtles *et al.*, 1996). The degree of 16S rRNA sequence similarity between LLAPs and validly described members of the genus *Legionella* was found to be consistent with that between the existing members of the genus. As with the phylogenetic dendrogram based on *mip* gene sequences (Fig. 1), unrooted consensus trees based on 16S rRNA sequence similarities (Adeleke *et al.*, 1996; Birtles *et al.*, 1996), showed that the LLAP strains studied formed a coherent cluster with other *Legionella* species. Phylogenetic analysis suggested that LLAP-1^T and LLAP-10^T, which each belonged to separate clades, represented two new species of *Legionella* and this was confirmed by DNA homology. LLAP-6^T, LLAP-7, LLAP-9 and *L. lytica* formed another clade that represented at least one new species. The sequence similarities and branching patterns within the clade suggested that LLAP-7, LLAP-9 and *L. lytica* may represent the same species, while LLAP-6^T may represent a separate species. DNA homology experiments also confirmed these results. On the strength of these phylogenetic findings and other supporting data, it was proposed that LLAPs be included in the Family *Legionellaceae* and genus *Legionella* (Adeleke *et al.*, 1996).

Description of *Legionella drozanskii* sp. nov.

Legionella drozanskii (dro′zan.ski.i. N.L. gen. n. *drozanskii* in honour of Wincenty Drozanski, who isolated the first known LLAP strain and pioneered LLAP research).

Gram-negative, catalase-positive and oxidase-negative. The organism requires L-cysteine for growth on BCYE and does not ferment glucose. The organism exists naturally as a parasite of free-living amoebae but can be grown on BCYE. Slightly convex colonies with entire edges are seen after 3–4 d incubation at 30 °C. No growth is observed at 35 °C. Cultures are initially grey to blue-grey and have a soft/light butterous consistency. With prolonged incubation, colonies appear to become less grey in colour, tending towards white. Colonies do not autofluoresce. Branched-chain CFAs predominate in cell wall. Although it contains ubiquinones with Q-10, Q-11 and Q-14 isoprene units in the polyprenoid side chain, the major ubiquinones are Q-12 and Q-13. The type strain is LLAP-1^T (= ATCC 700990^T).

Description of *Legionella rowbothamii* sp. nov.

Legionella rowbothamii (row.bo′tham.i.i. N.L. gen. n. *rowbothamii* in honour of Timothy Rowbotham, who isolated the majority of known LLAP strains and has contributed significantly to our knowledge of LLAPs).

Gram-negative, catalase-positive, oxidase-negative bacillus that requires L-cysteine for growth on BCYE and does not ferment glucose. Although it can be grown on artificial media, the organism exists naturally as a parasite of free-living amoebae. Although culturable at 35 °C, optimal growth is observed on BCYE after 3–4 d incubation at 30 °C. The colonies are slightly convex with entire edges and are initially grey to blue-grey with a butterous consistency. With prolonged incubation, the colonies acquire a yellow pigmentation and become slippery and adherent. Colonies exhibit blue-white autofluorescence. The cell wall is predominated by branched-chain CFAs and contains minor amounts of i-3-OH-15:0, a branched-chain ester-linked hydroxy acid. The major ubiquinones are Q-12 and Q-13 with smaller amounts of Q-10, Q-11 and Q-14 isoprene units in the polyprenoid side chain. The type strain is LLAP-6^T (= ATCC 700991^T).

Description of *Legionella fallonii* sp. nov

Legionella fallonii (fall′o.ni.i. N.L. gen. n. *fallonii* in honour of the Late Ronald Fallon who contributed immensely to our knowledge of legionellae and was the first to produce *Legionella* antigen and antiserum in the UK in 1977. He worked on an isolate from a case on the ship from which LLAP-10^T was isolated).

Gram-negative rod that does not ferment glucose. Catalase-positive, weakly oxidase-positive and requires L-cysteine for growth on BCYE. Exists naturally as a parasite of free-living amoebae but can be grown on BCYE. Optimal growth is observed after 3–4 d incubation at 30 °C. The convex colonies with entire edges are initially grey to blue-grey with a butterous consistency. However, with age, the smooth non-fluorescent colonies become beige to brown in colour. The cell wall contains relatively high amounts of branched-chain CFAs and the major ubiquinones are Q-12 and Q-13. It also contains smaller amounts of ubiquinones Q-10, Q-11 and Q-14 isoprene units in the

polyprenoid side chain. The type strain is LLAP-10^T (= ATCC 700992^T).

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