Five New *Legionella* Species Isolated from Water

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Fourteen *Legionella*-like strains isolated from aquatic sources have been characterized serologically, biochemically, and in terms of DNA relatedness. The strains grew on buffered charcoal-yeast extract agar but not on blood agar and displayed phenotypic characteristics typical of the family *Legionellaceae*, including a requirement for cysteine, cellular fatty acid compositions in which branched-chain acids predominate, and the possession of isoprenoid quinones of the ubiquinone series with more than 10 isoprene units in their side chains. All were nonfermentative, lacked urease, were incapable of nitrate reduction, and reacted positively with a DNA probe specific for the *Legionellaceae*. DNA hybridization studies in which the hydroxyapatite method was used demonstrated that the strains represented five new species of the genus *Legionella*. Nine of the strains were more than 90% interrelated, and the name *Legionella londiniensis* sp. nov. is proposed for this group. Two strains formed a second hybridization group, for which the name *Legionella nautarum* sp. nov. is proposed, while the three remaining species, *Legionella geestiana* sp. nov., *Legionella quateirensis* sp. nov., and *Legionella worsleiensis* sp. nov., are each represented by a single strain. The levels of relatedness of the new species to each other are 23% or less, and the levels of relatedness to other members of the genus ranged from 0 to 36%. *L. geestiana*, *L. nautarum*, and *L. londiniensis* are serologically unrelated to all other known *Legionella* species. *L. worsleiensis* cannot be separated from *Legionella pneumophila* serogroup 4 by serological methods and is also serologically indistinguishable from *L. quateirensis*; distinctions may be made on the basis of fatty acid composition and biochemical reactions.

It has now been 14 years since the classification of *Legionella pneumophila* as a new bacterium in the family *Legionellaceae* (11). Since this classification an additional 34 species of this genus have been described (1–3, 7, 10, 27–30, 32, 39, 40).

Between 1982 and 1984 the Public Health Laboratory Service in England and Wales undertook a survey of buildings, particularly hotels and hospitals, to determine the incidence of legionellae in their water systems and to identify the physical and chemical factors predisposing buildings to colonization. During this survey a number of strains were isolated that conformed to the definition of the family *Legionellaceae* but could not be assigned to previously described species. In this paper we describe the characterization of these strains and propose names for five new species isolated from water samples. To our knowledge none of these species has so far been implicated in human infections.

MATERIALS AND METHODS

Bacterial strains. Strains of the five new species are listed in Table 1. All strains were isolated by direct plating onto buffered charcoal-yeast extract (BCYE) media made selective by addition of glycine and antibiotics, using a standardized isolation protocol (13). Briefly, water samples were concentrated by membrane filtration, and the material re-

tained on each filter was resuspended in water and directly plated onto the selective media. Portions of the concentrates were also treated with an acid buffer (6) or heated to 50°C prior to plating (13). Strains were cultured and maintained on BCYE agar at 35 ± 1°C in air containing 2.5% CO₂. Suspensions were stored at –80°C.

Cultural and biochemical tests. All strains of the new species were tested by the methods described by Vesey et al. (33). These methods consist of modifications of procedures previously described for the differentiation of members of the *Legionellaceae*, together with some additional tests. All of the strains were stained by using the Preston-Morrell modification of Gram stain (24). The requirement for L-cysteine was investigated by plating cultures onto BCYE agar lacking this amino acid. Cellular fluorescence and pigment production on media supplemented with aromatic substrates were investigated by using modified versions of the methods described by Vickers and Yu (35). The bromocresol purple spot test was performed as described by Garrity et al. (15). Dye-containing media were prepared as described by Holmes (17) and Vickers et al. (34).

The following enzyme activities were tested for: elastase production, casein hydrolysis, albumin degradation, chondroitin sulfate degradation, starch hydrolysis, DNase activity, gelatin hydrolysis, lipase activity, and protease activity. Hydrolysis of sodium hippurate was tested by the method of Hébert (16). Combined peroxidase and catalase activities were measured by a modification of the procedure of Pine et al. (23). Esterase and alkaline phosphatase activities were determined by the methods described by Orrison et al. (22). β-Lactamase production was measured by using a chromogenic test with nitrocefin-impregnated discs (Beckton, Dick-

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TABLE 1. Bacterial strain information

Strain	Source	Location	G+C content (mol%)
<i>L. geestiana</i> 1308 ^T (= ATCC 49504 ^T) ^a	Domestic hot water tap	Geest Office Building, London, United Kingdom	52
<i>L. nautarum</i> 1224 ^T (= ATCC 49506 ^T)	Domestic hot water tap	Seamans Hospital, Greenwich, London, United Kingdom	41
<i>L. nautarum</i> 1231	Domestic hot water tap	Seamans Hospital, Greenwich, London, United Kingdom	
<i>L. londiniensis</i> 1477 ^T (= ATCC 49505 ^T)	Cooling tower pond water	Office building, London, United Kingdom	43
<i>L. londiniensis</i> 1466	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1468	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1471	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1472	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1473	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1474	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1475	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. londiniensis</i> 1476	Cooling tower pond water	Office building, London, United Kingdom	
<i>L. quateirensis</i> 1335 ^T (= ATCC 49507 ^T)	Shower in bathroom	Hotel, Quarteira, Portugal	39
<i>L. worsleiensis</i> 1347 ^T (= ATCC 49508 ^T)	Cooling tower return flow	Industrial site, Worsley, United Kingdom	41

^a ATCC, American Type Culture Collection.

inson and Co., Oxford, United Kingdom) which were moistened and wiped across a single colony. The oxidase test was performed by using a 0.5% suspension of tetramethyl *p*-phenylenediamine. Motility was determined by examining wet mounts of cultures grown in yeast broth (25) for 2, 3, and 4 days, using phase-contrast microscopy. Growth (if present) on Mueller-Hinton agar (38) and *Legionella* blood agar (14) was noted after 7 days of incubation at 35 ± 1°C in the presence of 2.5% CO₂.

SAT. Strains 1224^T (T = type strain) 1308^T, 1335^T, 1347^T, and 1477^T were used to prepare slide agglutination test (SAT) antigens and antisera (31). The five antisera were tested against reference strains of previously described legionellae (34 species and 53 serogroups) and against all of the strains listed in Table 1. Similarly, antigens prepared from these five strains were tested with antisera to all of the strains listed in Table 1. Antigens and antisera for two recently described *Legionella* serogroups, *Legionella quinlivanii* serogroup 2 and *Legionella erythra* serogroup 2 (4, 26), were not available for testing.

Extraction and analysis of cellular fatty acids. Strains were grown on BCYE media for 3 days at 35 ± 1°C in air containing 2.5% CO₂, harvested into distilled water, and freeze dried. Fatty acid methyl esters were prepared by acid methanolysis of the freeze-dried cells as previously described (36). Gas-liquid chromatography was performed with a Carlo-Erba model 4130 chromatograph fitted with a fused-silica BP-5 column (25 m by 0.2 mm; SGE Limited, Milton Keynes, United Kingdom). Helium was used as the carrier gas at a linear velocity of 30 cm s⁻¹. The samples (dissolved in trimethylpentane) were introduced by splitless injection (splitless time, 30 s) at an initial oven temperature of 80°C. After a 1-min delay the oven was programmed to increase its temperature to 210°C at a rate of 40°C min⁻¹, and this temperature was held for 20 min. Peaks were identified by their relative retention times and by gas chromatography-mass spectrometry, using a model MS80 RFA mass spectrometer (Kratos Ltd., Manchester, United Kingdom) interfaced with a Carlo-Erba model 5160 chromatograph. The chromatographic conditions were the same as those described above, except that a BP-1 column (25 m by 0.2 mm) was used. The instrument was operated in electron impact

mode at an ionization energy of 70 e V, a trap current of 100 µA, and a scan rate of 0.3 s per decade of mass.

Extraction and analysis of isoprenoid quinones. Isoprenoid quinones were extracted by the method of Collins et al. (12). Between 20 and 50 mg of freeze-dried cells was suspended in 20 ml of chloroform-methanol (2:1) and magnetically stirred for 2 h at room temperature. The mixture was filtered (Whatman no. 1 filter paper), and the filtrate was dried by rotary evaporation and purified by thin-layer chromatography on silica gel 60F₂₅₄ plates (BDH Ltd., Poole, United Kingdom). The plates were developed with hexane-diethyl ether (85:15), and the quinone-containing band was visualized under UV light (254 nm) and scraped off. Quinones were eluted from the silica with 5 ml of chloroform and filtered through a sintered glass funnel, and the chloroform was evaporated in a stream of nitrogen. The purified quinones were analyzed by reversed-phase thin-layer chromatography, high-performance liquid chromatography (HPLC), and mass spectrometry.

Reverse-phase high-performance thin-layer chromatography (RP-HPTLC) was performed by using HPTLC 18F₂₅₄ plates (10 by 10 cm; BDH) developed in acetone-acetonitrile (80:20). The separated quinones were visualized by viewing the developed plates under UV light (254 nm). The HPLC system consisted of two model 510 pumps, an automatic gradient controller, a model 710B (Waters) intelligent sample processor (WISP), a model 481 LC spectrophotometer, and a model 740 data module (all obtained from Waters Associates). The column was a µBondapak C₁₈ radial compression cartridge, used in conjunction with a model RCM 100 radial compression module. The mobile phase was methanol-1-chlorobutane (70:30) at a flow rate of 1.5 ml min⁻¹. The quinone samples were dissolved in 200 µl of 1-chlorobutane, and the WISP was programmed to inject 15 µl. The eluted quinones were detected by their UV absorption at 270 nm.

Negative-ion fast atom bombardment mass spectra were recorded with a model MS80 RFA mass spectrometer (Kratos) equipped with a model BN11F saddle field gun (Ion Tech Ltd., Teddington, United Kingdom) using xenon as the bombarding gas. The accelerating voltage was 4 k V, and the magnet was scanned at 30 s decade⁻¹ from a starting mass of 1,600. Quinone samples (1 to 10 µg) in 1 µl of chloroform

TABLE 2. DNA relatedness of the new *Legionella* species

Source of labelled or unlabelled DNA ^a	DNA relatedness to labelled or unlabelled DNA from:								
	<i>L. nautarum</i> 1224 ^T			<i>L. geestiana</i> 1308 ^T : % relatedness at 60°C	<i>L. quateirensis</i> 1335 ^T : % relatedness at 60°C	<i>L. worsleiensis</i> 1347 ^T : % relatedness at 60°C	<i>L. londiniensis</i> 1477 ^T		
	% Relatedness at 60°C	Divergence	% Relatedness at 75°C				% Relatedness at 60°C	Divergence	% Relatedness at 75°C
<i>L. nautarum</i> 1224 ^T	100	0.0	100		6				
<i>L. nautarum</i> 1231	70	0.0	78						
<i>L. geestiana</i> 1308 ^T	4			100	11	3			
<i>L. quateirensis</i> 1335 ^T	2			6	100	13			
<i>L. worsleiensis</i> 1347 ^T	4			11	23	100	3		
<i>L. londiniensis</i> 1477 ^T	2			3	6	4	100	0.0	100
<i>L. londiniensis</i> 1466							94	1.0	100
<i>L. londiniensis</i> 1468							99	0.0	99
<i>L. londiniensis</i> 1471							98	0.0	87
<i>L. londiniensis</i> 1472							97	0.5	100
<i>L. londiniensis</i> 1473							100	0.0	100
<i>L. londiniensis</i> 1474							94	0.0	97
<i>L. londiniensis</i> 1475							98	0.5	98
<i>L. londiniensis</i> 1476							95	0.5	95
<i>L. pneumophila</i> Philadelphia 1 ^T	7			2	12	6	4		
<i>L. bozemanii</i> WIGA ^T	6			3	8	4	4		
<i>L. micdadei</i> TATLOCK ^T	24			1	8	8	0		
<i>L. dumoffii</i> NY-23 ^T	9			4	13	4	3		
<i>L. gormanii</i> LS-13 ^T	19			5	12	4	0		
<i>L. longbeachae</i> Long Beach 4 ^T	9			4	10	2	7		
<i>L. jordanis</i> BL-540 ^T	8			6	2	5	3		
<i>L. oakridgensis</i> Oak Ridge 10 ^T	5			3	5	2	12		
<i>L. wadsworthii</i> 81-716A ^T	9			6	11	3	8		
<i>L. feeleeii</i> WO-44C-C3 ^T	14			3	7	2	5		
<i>L. sainthelensi</i> Mount Saint Helens 4 ^T	6			4	7	2	3		
<i>L. anisa</i> WA-316-C3 ^T	4					6			
<i>L. maceachernii</i> PX-1-G2-E2 ^T	9			5	9	2	0		
<i>L. jamestowniensis</i> JA-26-G1-E2 ^T	11			4	8	2	5		
<i>L. rubrilucens</i> WA-270A-C2 ^T	1			6		3	6		
<i>L. erythra</i> SE-32A-C8 ^T	9			10	10	6	9		
<i>L. hackeliae</i> Lansing 2 ^T	11			4	7	6	8		
<i>L. spiritensis</i> Mount Saint Helens 9 ^T	8			6	9	1	6		
<i>L. parisiensis</i> PF-209C-C2 ^T	10			2	11	5	2		
<i>L. cherrii</i> ORW ^T	2					2			
<i>L. steigerwaltii</i> SC-18-C9 ^T	0					3			
<i>L. santicrucis</i> SC-63-C7 ^T	2					2			
<i>L. israelensis</i> Bercovier 4 ^T	4			4	5	3	8		
<i>L. birminghamensis</i> 1407-AL-H ^{Tb}	1			2	2	1	2		
<i>L. cincinnatiensis</i> 72-OH-H ^{Tb}	2			3	4	3	1		
<i>L. brunensis</i> 441-1 ^{Tb}	3			0	2	2	1		
<i>L. moravica</i> 316-36 ^{Tb}	2			3	36	24	1		
<i>L. quinlivanii</i> 1441-AUS-E ^{Tb}	2			1	4	2	2		
<i>L. tucsonensis</i> 1087-AZ-H ^{Tb}	10			6	11	12	3		
<i>L. gratiana</i> Lyon 8420412 ^{Tb}	3			4	8	6	3		
<i>L. fairfieldensis</i> 1725-AUS-E ^{Tb}	9			1	10	8	5		
<i>L. adelaidensis</i> 1762-AUS-E ^{Tb}	2			3	3	1	2		
<i>L. shakespearei</i> 214 ^{Tb}	3			2	9	7	3		
<i>L. lansingensis</i> 1677-MI-H ^{Tb}	2			1	2	2	1		

^a Source of unlabelled DNA except as noted below.^b Source of labelled DNA.

were mixed with 1 µl of 3-nitrobenzyl alcohol matrix on the stainless steel fast atom bombardment target. Between 5 and 10 scans were acquired and averaged by using the raw data facilities of a model DS90 data system. Cesium iodide was used as the mass calibrant.

DNA studies. The guanine-plus-cytosine (G+C) content of DNA was determined with a spectrophotometer by thermal denaturation (20). Preparation of DNA and use of the hydroxyapatite method for DNA hybridization have been

described previously (9). In vitro labelling of DNA was performed enzymatically by using [³²P]dCTP provided in a nick translation reagent kit (catalog no. 810608B; Bethesda Research Laboratories, Inc., Gaithersburg, Md.), as directed by the manufacturer. Percentages of divergence in related DNA sequences were determined to the nearest 0.5% by thermal elution of DNA from hydroxyapatite, on the assumption that each degree of diminished thermal stability in a heteroduplex, compared with the stability of a homolo-

gous DNA duplex, was caused by 1% unpaired bases (9). A ^3H -labelled *Legionella*-specific DNA probe (18) was used under standard hybridization conditions. The probe was a gift from D. E. Kohne (Gen-Probe Inc., San Diego, Calif.).

RESULTS

Putative *Legionella* isolates from the environmental sampling study were investigated by using routine biochemical and serological tests. A total of 17 strains could not be identified. DNA hybridization studies subsequently showed that one of these strains was a *Legionella pneumophila* strain and two others were strains of the newly described species *Legionella rubrilucens* (10).

DNA hybridization. The 14 remaining strains were tested to determine their levels of DNA relatedness to each other and to known *Legionella* species (Table 2). In some cases the new strains were not tested against *Legionella anisa*, *L. rubrilucens*, *Legionella cherrii*, *Legionella steigerwaltii*, and *Legionella santacrucis*, since each of these species is substantially related to one or more of the other species (10) against which the new strains were tested.

The new strains formed five hybridization groups, which were 0 to 36% related to known *Legionella* species. The reference strain from each group hybridized with a *Legionella*-specific DNA probe (data not shown). Strain 1477^T and eight other strains were more than 90% related; the name *Legionella londiniensis* sp. nov. is proposed for these organisms. Strains 1224^T and 1231 formed a second hybridization group, for which the name *Legionella nautarum* sp. nov. is proposed. Each of the three remaining strains comprised a separate hybridization group. The name *Legionella geestiana* sp. nov. is proposed for strain 1308^T, the name *Legionella quateirensis* sp. nov. is proposed for strain 1335^T, and the name *Legionella worsleiensis* sp. nov. is proposed for strain 1347^T.

SAT. No cross-reactions were observed with antigens of the two strains of *L. nautarum* and the nine *L. londiniensis* strains when they were tested with antisera prepared against members of the previously described 34 species and 53 serogroups of the genus *Legionella* or with antisera prepared against *L. geestiana* 1308^T, *L. quateirensis* 1335^T, and *L. worsleiensis* 1347^T. The cross-reactions observed with *L. geestiana*, *L. worsleiensis*, and *L. quateirensis* antigens are listed in Table 3. Absorption of *Legionella jamestowniensis*

antiserum with *L. geestiana* cells removed agglutinating antibodies against *L. geestiana* but not agglutinating antibodies against *L. jamestowniensis*. Two of the antisera which cross-reacted with *L. worsleiensis* cells, the *L. pneumophila* serogroup 4 strain Portland 1 and *L. quateirensis* antisera, could not be made serogroup specific. Absorption of these antisera with *L. worsleiensis* cells removed all agglutinating antibodies for both the heterologous and the homologous strains. *L. quateirensis* antigen also cross-reacted with several *Legionella* antisera. However, these cross-reactions were removed by absorbing the antisera with *L. quateirensis* cells.

The results of testing antisera prepared against the five new species with homologous and heterologous SAT antigens are presented in Table 4. Antisera prepared against the type strains of *L. nautarum*, *L. londiniensis*, and *L. geestiana* were negative with all heterologous *Legionella* antigens. *L. nautarum* 1224^T antiserum reacted 4+ with both *L. nautarum* strains, and *L. londiniensis* 1477^T antiserum reacted 4+ with all nine *L. londiniensis* strains. Antiserum against *L. worsleiensis* 1347^T, at its optimal working dilution of 1:16, cross-reacted 4+ with *L. pneumophila* serogroup 4 strain Portland 1 and *L. quateirensis* cells and 2+ with *L. pneumophila* serogroup 8 strain Concord 3 cells. Absorption of 1347^T antiserum with 1335^T cells removed agglutinating antibodies to strain 1335^T but not agglutinating antibodies to strain Portland 1. Further absorption of 1347^T antiserum with Portland 1 cells removed all agglutinating antibodies to both strain Portland 1 and the homologous strain.

At its optimal working dilution of 1:16, *L. quateirensis* antiserum cross-reacted 4+ with *L. pneumophila* serogroup 4 strain Los Angeles 1, *L. pneumophila* serogroup 10 strain Leiden 1, *L. pneumophila* serogroup 13 strain 82A3105, and 1347^T cells, and its showed 2+ and 1+ agglutination with *L. pneumophila* serogroup 4 strain Portland 1 and serogroup 12 strain 570-CO-H, respectively. Absorption of *L. quateirensis* antiserum with *L. pneumophila* serogroup 13 strain 82A3105 cells removed all heterologous agglutinating antibodies except the *L. worsleiensis* 1347^T antibodies. Further absorption of the 1335^T antiserum with 1347^T cells removed all heterologous (1347^T) and homologous (1335^T) agglutinating activities.

Cellular fatty acid composition. The members of the *Legionellaceae* are characterized (unusually for gram-negative organisms) by the possession of high levels of branched-

TABLE 3. Cross-reactions observed with unabsorbed *Legionella* antisera in the SAT

<i>Legionella</i> antigen			<i>Legionella</i> antiserum (unabsorbed)	Cross-reaction
Species	Serogroup	Strain		
<i>L. geestiana</i>	1	1308 ^T	<i>L. jamestowniensis</i> JA-26-G1-E2 ^T	3+ ^a
<i>L. worsleiensis</i>	1	1347 ^T	<i>L. pneumophila</i> serogroup 4 strain Portland 1	4+
			<i>L. pneumophila</i> serogroup 5 strain Dallas 1	4+
			<i>L. pneumophila</i> serogroup 8 strain Concord 3	4+
			<i>L. pneumophila</i> serogroup 10 strain Leiden 1	4+
			<i>L. quateirensis</i> serogroup 1 strain 1335 ^T	4+
<i>L. quateirensis</i>	1	1335 ^T	<i>L. pneumophila</i> serogroup 5 strain Dallas 1	4+
			<i>L. pneumophila</i> serogroup 10 strain Leiden 1	4+
			<i>L. worsleiensis</i> serogroup 1 strain 1347 ^T	4+
			<i>L. pneumophila</i> serogroup 4 strain Portland 1	2+
			<i>L. pneumophila</i> serogroup 8 strain Concord 3	2+
			<i>L. pneumophila</i> serogroup 13 strain 82A3105	2+
			<i>L. pneumophila</i> serogroup 14 strain 1169-MN-H	2+
			<i>L. pneumophila</i> serogroup 4 strain Los Angeles 1	1+

^a Positive reactions were scored on a scale from 1+ (barely visible) to 4+ (strong agglutination).

TABLE 4. Preparation of *Legionella* antisera for the SAT

Immunizing antigen			SAT titer ^a	Antiserum	Cross-reaction ^b
Species	Serogroup	Strain			
<i>L. nautarum</i>	1	1224 ^T	1:32	None	
<i>L. londiniensis</i>	1	1477 ^T	1:16	None	
<i>L. geestiana</i>	1	1308 ^T	1:8	None	
<i>L. worsleiensis</i>	1	1347 ^T	1:16	<i>L. pneumophila</i> serogroup 4 strain Portland 1	4+
				<i>L. quateirensis</i> serogroup 1	4+
				<i>L. pneumophila</i> serogroup 8	2+
<i>L. quateirensis</i>	1	1335 ^T	1:16	<i>L. pneumophila</i> serogroup 4 strain Los Angeles 1	4+
				<i>L. pneumophila</i> serogroup 10	4+
				<i>L. pneumophila</i> serogroup 13	4+
				<i>L. worsleiensis</i> serogroup 1	4+
				<i>L. pneumophila</i> serogroup 4	2+
				<i>L. pneumophila</i> serogroup 12	1+

^a Highest dilution giving 4+ agglutination.^b Intensities of cross-reactions observed by using working dilutions of antisera.

chain fatty acids, particularly 14-methylpentadecanoic acid (i16:0), 14-methylhexadecanoic acid (a17:0), and 12-methyltetradecanoic acid (a15:0). The fatty acid compositions of the five new species (Table 5) are thus consistent with those of other members of the genus (19).

The fatty acid profiles of *L. quateirensis*, *L. nautarum*, and *L. worsleiensis* are similar to each other and to the fatty acid profile of *Legionella feeleyi* in that the proportion of unbranched acids (particularly 16:0, 16:1, and 18:0) is higher than this proportion in most *Legionella* species. The relative proportion of i16:0 and 16:1 was somewhat variable from experiment to experiment in these three organisms, a phenomenon which has also been observed in *L. feeleyi* (21), where the fatty acid composition was shown to depend on the age of the culture. *L. worsleiensis* and *L. quateirensis* lack the cyclopropane ring-containing compound 9,10-methylenehexadecanoic acid (cyc-17).

L. londiniensis also lacks cyc-17 but is distinguishable from the other species because a17:0 is the predominant acid. *L. geestiana* differs from all previously described species by the presence of i15:0 and i17:0 in higher concentrations than their anteiso-branched isomers. 9,10-methylenehexadecanoic acid is again absent. There was also a peak not present in the other four species; this peak eluted before i17:0 and had a retention time similar to, but slightly shorter than, the retention time of a17:1 from *Legionella micdadei*. The mass spectrum of this constituent contained a molecular ion at *m/z* 282, suggesting that it was an unsaturated 17-carbon acid. Signals at *m/z* 227 ($M-C_4H_8$)⁺, *m/z* 195 (loss of CH₃OH from *m/z* 227), and *m/z* 177 (dehydration product of *m/z* 195) define the structure as 15-methylhexadecenoic acid (i17:1) (5). This acid is not usually encountered in other

legionellae and is thus potentially useful for species discrimination.

Isoprenoid quinone composition. Isoprenoid quinones of the ubiquinone series containing more than 10 isoprene units in the side chain are highly characteristic of the *Legionellaceae* (10, 19). Ubiquinones were the sole respiratory quinones detected by reverse-phase high-performance thin-layer chromatography, reverse-phase HPLC, and negative-ion fast atom bombardment mass spectrometry (37). The fast atom bombardment mass spectra were characterized by intense semiquinone molecular anion radicals [M]⁻, revealing the mass of each quinone molecular species present. A typical spectrum is shown in Fig. 1. The relative intensities of the molecular anions approximately reflect the concentrations of the individual quinones in the mixture and were in reasonable agreement with the results of the HPLC analysis (Table 6).

With the exception of the *L. londiniensis* profile, the quinone profiles were rather simple and were dominated by one or two major compounds. The quinone compositions of *L. worsleiensis*, *L. quateirensis*, and *L. nautarum* are qualitatively similar, containing mainly Q13 (60 to 80%) and lesser amounts of Q12 and Q14. They thus somewhat resemble *L. micdadei*, *Legionella jordanis*, and *L. jamestowniensis* profiles (19, 36), although the former species differ from the latter species in fatty acid composition and other phenotypic characteristics. *L. geestiana* contains Q14 as its major quinone (approximately 50%), as well as Q13 (>40%); the remainder of the quinone complement consists of Q12 and Q15. This is rather similar to the *L. feeleyi* profile, but again the fatty acid composition of *L. geestiana* makes confusion very unlikely. The quinone profile of *L. londiniensis* is

TABLE 5. Major nonhydroxylated fatty acids of the new *Legionella* species

Strain	Fatty acid composition (% of total integrated area) ^a														
	i14:0	i15:0	a15:0	15:1	15:0	i16:1	i16:0	16:1	16:0	i17:1	i17:0	a17:0	cyc-17:0	17:0	18:0
<i>L. worsleiensis</i> 1347 ^T	4	tr	12	2	2	1	9	35	16	0	0	5	0	2	9
<i>L. quateirensis</i> 1335 ^T	3	tr	8	4	1	tr	20	35	7	0	0	4	0	3	10
<i>L. londiniensis</i> 1477 ^T	tr	tr	5	2	1	tr	11	19	15	0	0	23	0	8	8
<i>L. nautarum</i> 1224 ^T	2	0	19	2	2	tr	9	27	19	0	0	13	4	1	2
<i>L. geestiana</i> 1308 ^T	tr	16	9	1	1	0	2	20	18	2	9	7	0	1	4

^a Values are rounded off to the nearest whole percent. The total may not equal 100% because of rounding off and the omission of minor components. tr, trace (<1%).

FIG. 1. Molecular ion region of the negative-ion fast atom bombardment mass spectrum of the ubiquinones of *L. londiniensis* 1466. The signals at m/z 862, 930, and 998 correspond to the molecular anions of Q10, Q11, and Q12, respectively. Each is accompanied by a fragment ion 15 a.m.u. lower in mass because of the elimination of a methyl group.

dominated by Q11 (approximately 60% of the total); Q10 and Q12 make up most of the balance (Fig. 1). *L. londiniensis* thus differs from the other four new species, and its quinone composition is also unlike any of the quinone compositions reported for 23 species by Lambert and Moss (19); it does however bear some resemblance to the quinone profile of the recently described species *Legionella adelaidensis*, which is also dominated by Q11 (1).

Biochemical tests. The biochemical characteristics of the five new species are shown in Table 7 and are detailed in the species descriptions below. *L. quateirensis* and *L. worsleiensis*, which are not serologically distinguishable, are separable biochemically, since *L. quateirensis* hydrolyzes

starch and is positive for catalase, lipase, and protease activities, whereas *L. worsleiensis* is not.

DISCUSSION

Fourteen *Legionella*-like strains were shown to belong to the genus *Legionella* on the basis of growth characteristics, biochemical reactions, cell wall fatty acid and isoprenoid quinone compositions, and hybridization of their DNAs with a *Legionella*-specific gene probe. All strains required cysteine for growth and therefore grew on BCYE medium, but not on blood or Mueller-Hinton media. They were nonfermentative, lacked urease, and did not reduce nitrate. They contained predominantly branched-chain cell wall fatty acids and major amounts of ubiquinones with more than 10 isoprene units. Positive reactions with a DNA probe specific for the genus *Legionella* confirmed that these strains properly belong in the family *Legionellaceae* as presently constituted with the single genus *Legionella*.

These 14 strains formed five DNA relatedness groups that were less than 23% interrelated and showed 36% or lower levels of relatedness to previously described *Legionella* species. These data confirm that each of the DNA relatedness groups represents a new *Legionella* species. Three of the new species, *L. geestiana*, *L. nautarum*, and *L. londiniensis*, are serologically unique. *L. worsleiensis* and *L. quateirensis* cannot be serologically separated from each

TABLE 6. Ubiquinone compositions of the five new species

Strain	Ubiquinone composition (% of total integrated area) ^a						
	Q9	Q10	Q11	Q12	Q13	Q14	Q15
<i>L. londiniensis</i> 1466	2	24	55	16	2	tr	ND
<i>L. worsleiensis</i> 1347 ^T	ND	1	4	26	62	4	tr
<i>L. quateirensis</i> 1335 ^T	ND	tr	2	21	69	8	tr
<i>L. nautarum</i> 1224 ^T	ND	ND	ND	18	76	6	ND
<i>L. geestiana</i> 1308 ^T	ND	ND	1	4	39	52	2

^a The masses of the [M]⁻ ions were as follows: Q10, 862; Q11, 930; Q12, 998; Q13, 1,066; and Q14, 1,134. tr, trace (<1%); ND, not detected.

TABLE 7. Biochemical characteristics of the new *Legionella* species

Reaction	<i>L. worsleiensis</i>	<i>L. quateirensis</i>	<i>L. geestiana</i>	<i>L. londiniensis</i>	<i>L. nautarum</i>
Hippurate hydrolysis	— ^a	—	+ ^w	— or + ^w	—
D-Glucose (acid production)	—	—	—	—	—
Catalase activity	—	+	+	+ ^w	+
Oxidase activity	—	—	—	—	+
Peroxidase activity	+	+	+ ^w	—	+
Urease activity	—	—	—	—	—
Browning with the following substrates:					
Tyrosine	+	+	+ ^w	+	—
3,4-Diaminobenzoic acid	+ ^w	+	—	—	—
3,5-Diaminobenzoic acid	+	+	—	—	—
Autofluorescence	—	—	—	—	—
Bromocresol purple spot test	—	—	—	—	—
β-Lactamase activity	+	+	—	+	+
Elastase production	—	—	+	+	—
Casein hydrolysis	+	+	+	+ ^w	—
Albumin degradation	+	+	+	+ ^w	—
Chondroitin sulfate degradation	+	+	+	—	—
Starch hydrolysis	—	+ ^w	—	—	—
DNase activity	+	+	+	+	—
Gelatin hydrolysis	+	+	+	+	—
Lipase activity	—	+	—	—	+
Protease activity	—	+	—	—	—
Alkaline phosphatase activity at:					
pH 9.5	+	+	+	+	+
pH 10.5	+	+	+	+	+
Single polar flagellum	+	+	+	—	—
Requirement for cysteine	+	+	+	+	+
Growth on:					
<i>Legionella</i> blood agar	—	—	—	—	—
Mueller-Hinton agar	—	—	—	—	—
BCYE agar	+	+	+	+	+
Nitrate reduction	—	—	—	—	—

^a +, positive; —, negative; +^w, weakly positive.

other, but may be distinguished by their biochemical reactions (Table 7). *L. worsleiensis* and *L. pneumophila* serogroup 4 are likewise serologically indistinguishable but may be differentiated by their fatty acid profiles and biochemically, since *L. pneumophila* serogroup 4 is positive for hippurate and starch hydrolysis and negative for browning on 3,4- and 3,5-diaminobenzoic acids (33), whereas the converse is true of *L. worsleiensis* (Table 7).

Taxonomic proposals. Nomenclatural proposals are given below for five new species of the genus *Legionella* that conform to the definitions of the family *Legionellaceae* and the genus *Legionella* (8, 10). All of the species are gram-negative rods that require cysteine for growth and grow on BCYE agar but not on blood agar or Mueller-Hinton agar. They are not autofluorescent and do not reduce nitrate, contain a urease, or produce acid from D-glucose. They exhibit alkaline phosphatase activity at pH 9.5 and 10.5 and are negative for the bromocresol purple spot test. The sources of the strains of the species are given in Table 1, and their DNA relatedness relationships are shown in Table 2. Tables 5 through 7 summarize their fatty acid profiles, quinone compositions and biochemical reactions. Their serological reactions are listed in Tables 3 and 4. Other characteristics are given above and below.

***Legionella geestiana* sp. nov.** *Legionella geestiana* (gees. ti. a' na. N.L. adj. *geestiana*, pertaining to the Geest office building) is an oxidase-negative, catalase-positive organism with a single polar flagellum. It reacts positively in tests for hippurate hydrolysis, peroxidase activity, browning on agar containing tyrosine, elastase production, casein hydrolysis,

albumin degradation, chondroitin sulfate degradation, DNase activity, and gelatin hydrolysis. It gives negative results in tests for browning on agar containing 3,4- and 3,5-diaminobenzoic acid, β-lactamase activity, starch hydrolysis, lipase activity, and protease activity. The type strain is strain 1308 (= ATCC 49504), which has a G+C content of 52 mol%.

***Legionella nautarum* sp. nov.** *Legionella nautarum* (nau. tar' um. N.L. gen. n. *nautarum*, of sailors) is a nonmotile oxidase- and catalase-positive organism. It exhibits positive reactions in tests for peroxidase, β-lactamase, and lipase activities. It gives negative results in tests for hippurate hydrolysis, elastase production, casein hydrolysis, albumin degradation, chondroitin sulfate degradation, starch hydrolysis, DNase activity, gelatin hydrolysis, protease activity, and browning on agar containing tyrosine, 3,4-diaminobenzoic acid, and 3,5-diaminobenzoic acid. The type strain is strain 1224 (= ATCC 49506), which has G+C content of 41 mol%.

***Legionella londiniensis* sp. nov.** *Legionella londiniensis* (lon. din. i. en' sis. N.L. gen. n. *londiniensis*, coming from London) is a nonmotile, oxidase-negative, catalase-positive organism. It gives positive reactions in tests for browning on agar containing tyrosine, β-lactamase activity, elastase production, and casein hydrolysis. It exhibits negative reactions in tests for hippurate hydrolysis (sometimes weakly positive), peroxidase activity, browning on agar containing 3,4- or 3,5-diaminobenzoic acid, chondroitin sulfate degradation, starch hydrolysis, lipase activity, and protease activity. The

type strain is strain 1477 (= ATCC 49505), which has a G+C content of 43 mol%.

Legionella quateirensis sp. nov. *Legionella quateirensis* (qua. teir. en' sis. N.L. gen. n. *quateirensis*, coming from Quarteira) is an oxidase-negative, catalase-positive organism with a single polar flagellum. It gives positive results in tests for peroxidase activity, browning on agar containing tyrosine or 3,4- or 3,5-diaminobenzoic acid, β -lactamase activity, casein hydrolysis, albumin degradation, chondroitin sulfate degradation, starch hydrolysis, DNase activity, gelatin hydrolysis, lipase activity, and protease activity. It gives negative reactions in tests for hippurate hydrolysis and elastase production. The type strain is strain 1335 (= ATCC 49507), which has a G+C content of 39 mol%.

Legionella worsleiensis sp. nov. *Legionella worsleiensis* (wors. lei. en' sis. N.L. gen. n. *worsleiensis*, coming from Worsley) is an oxidase- and catalase-negative organism with a single polar flagellum. It gives positive reactions in tests for peroxidase activity, browning on agar containing tyrosine or 3,4- or 3,5-diaminobenzoic acid, β -lactamase activity, casein hydrolysis, albumin degradation, chondroitin sulfate degradation, DNase activity, and gelatin hydrolysis. It gives negative reactions in tests for hippurate hydrolysis, elastase production, starch hydrolysis, lipase activity, and protease activity. The type strain is strain 1347 (= ATCC 49508), which has a G+C content of 41 mol%.

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