***Legionella gresilensis* sp. nov. and *Legionella beliardensis* sp. nov., isolated from water in France**

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Novel *Legionella*-like isolates, strains Montbéliard A1^T and Gréoux 11 D13^T, isolated from two different French water sources, were studied taxonomically and phylogenetically. Morphological and biochemical characterization revealed that they were Gram-negative, aerobic, non-spore-forming bacilli with a cut-glass appearance that grew only on L-cysteine-supplemented buffered charcoal yeast extract agar. Phenotypic characterization using fatty acid and ubiquinone profiles and SDS-PAGE analysis confirmed that they were closely related, but distinct from, other species of the genus *Legionella*, since serotyping could not relate them to any existing serogroup. Genotypic profiles generated by randomly amplified polymorphic DNA and 16S–23S rDNA spacer region PCR analyses were unique for each of these isolates. DNA–DNA relatedness values of strains Montbéliard A1^T and Gréoux 11 D13^T to each other and to other *Legionella* type strains were less than 25%. Phylogenetic affiliation of these organisms obtained by 16S rDNA sequence comparisons confirmed that they were distinct from any other known *Legionella* species. All the above results confirm that these strains constitute two novel species for which the names *Legionella gresilensis* sp. nov. (type strain Gréoux 11 D13^T = ATCC 700509^T = CIP 106631^T) and *Legionella beliardensis* sp. nov. (type strain Montbéliard A1^T = ATCC 700512^T = CIP 106632^T) are proposed.

Keywords: *Legionella gresilensis* sp. nov., *Legionella beliardensis* sp. nov., taxonomy, identification

INTRODUCTION

In 1976, a severe outbreak of pneumonia among veterans of the American Legion, in the city of Philadelphia, led to the discovery of a new genus

subsequently named *Legionella*. In 1979, this genus was composed of a single species, *Legionella pneumophila* (Brenner *et al.*, 1979). Since then, the genus has become very large, with 43 species currently recognized (Lo Presti *et al.*, 1999; Franzin & Gioannini, 2000). These bacteria have been isolated from a large diversity of samples: human clinical specimens, animals (Fabbri *et al.*, 1998), natural and man-made water samples (Fliermans, 1996; Fliermans *et al.*, 1981; Stout *et al.*, 1985), and protozoa (Newsome *et al.*, 1998). However, some species, such as *Legionella lytica* and other *Legionella*-like amoebal pathogens (LLAPs), remain unculturable on the buffered charcoal/yeast extract (BCYE) medium normally used to grow legionellae and on all other media tested (Adeleke *et al.*, 1996; Hookey *et al.*, 1996).

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Abbreviations: DFA, direct immunofluorescence assay; LLAPs, *Legionella*-like amoebal pathogens; RAPD, randomly amplified polymorphic DNA; SAT, slide agglutination test.

The GenBank accession numbers for the 16S rDNA sequences of strains Gréoux 11 D13^T, Montbéliard A1^T, ATCC 33737^T and ATCC 51914^T are AF122883–AF122886, respectively.

Routine identification of these bacteria is based on phenotypic properties (biochemical tests, serotyping), but molecular biology techniques have resulted in the development of new methods giving a more reliable characterization of *Legionella* species. Genotyping methods such as ribotyping (Grimont *et al.*, 1989), 16S–23S intergenic spacer region PCR (Riffard *et al.*, 1998), 16S or 5S rRNA gene sequencing (Fry *et al.*, 1991; Adeleke *et al.*, 1996; Birtles *et al.*, 1996; Hookey *et al.*, 1996; Murdoch *et al.*, 1999), macrophage infectivity potentiator (*mip*) gene sequencing (Ratcliff *et al.*, 1998) and randomly amplified polymorphic DNA (RAPD) (Lo Presti *et al.*, 1998) have been shown to be useful in classifying isolated strains to the species level. These methods are used to supplement DNA relatedness studies, which are used to confirm the designation of strains as novel species. Strains that cannot be recovered onto BCYE can be first characterized by 16S rDNA sequencing, which allows them to be classified as legionellae.

Routine investigations of French water from different origins (hospital and thermal spa) frequently lead to the isolation of untypable isolates. After phenotypic characterization, isolates remaining unidentified have been completely typed by genotypic methods. These experiments have allowed identification of two novel species, *Legionella beliardensis* sp. nov. and *Legionella gresilensis* sp. nov., isolated from different French water samples.

METHODS

Isolation procedure. Water samples (1 l) were collected from three different locations in France: a thermal spa in the city of Gréoux-les-Bains, a potash mine near Mulhouse and a hospital in Montbéliard. These samples were concentrated by continuous centrifugation and/or filtration to a 10 ml final volume and either left untreated or treated by acidification according to previously described procedures (Bornstein *et al.*, 1989). The resulting samples were cultured on: BCYE agar plates supplemented with 0.1% 2-oxoglutarate (BCYE α); BCYE α supplemented with glycine (3 g l⁻¹), vancomycin (5 mg l⁻¹) and colistin (5 mg l⁻¹) (GVC medium); and BAB agar which is a BCYE medium supplemented with colistin (16 mg l⁻¹), vancomycin (0.5 mg l⁻¹) and cefalothin (4.23 mg l⁻¹). Controls were done

on blood agar and on BCYE α without both L-cysteine and iron. The plates were incubated at 37 °C in an 80% H₂O atmosphere, with or without 2.5% CO₂.

Bacterial strains. The eight atypical *Legionella* isolates (Table 1) were compared with the species and serogroup type strains of 42 *Legionella* species and 65 serogroups (Table 3). Included in this study were some potentially novel species. The as yet not formally described '*Legionella donaldsonii*' (strain 'Glasgow' 86/35784; a gift from T. G. Harrison, Colindale, UK) and strain IB V no. 2 were also compared with our strains. As amoebal cultures were not available, one validly published species, *Legionella lytica*, could not be used for the DNA hybridization studies (Hookey *et al.*, 1996).

Cultural and biochemical tests. Each strain tested was cultured onto BCYE α or GVC medium when required. Requirement for cysteine on subculture was tested by its omission when preparing the BCYE α media. Auto-fluorescence of the strains was tested by examining cultures under Wood's light (366 nm). Tests for catalase, oxidase, β -lactamase, hippurate hydrolysis, urease, glucose utilization, gelatinase, nitrate reduction and presence of flagella were done as previously described (Brenner *et al.*, 1985). Browning of tyrosine-supplemented agar was considered positive if the browning produced around the colonies was larger on tyrosine-supplemented agar than any browning produced around the colonies of the same strain on tyrosine-free agar after 3 d growth (Vickers & Yu, 1984). The colour of the colonies was also noted on plates containing DGVP agar, a GVP medium modified by the addition of dyes (bromocresol purple and bromothymol blue) allowing a visual differentiation between *Legionella* (*Tatlockia*) *micdadei*, *Legionella* (*Tatlockia*) *maceachernii* (colonies are blue) and the other species of *Legionella* (colonies are green) (Vickers *et al.*, 1987).

Direct immunofluorescence assays. The eight *Legionella* isolates were identified by performing direct immunofluorescence assays (DFA) using 60 hyperimmune rabbit antisera prepared as described previously (Cherry *et al.*, 1978). Antisera were not available for four species: *Legionella* genomospecies 1, *Legionella lansingensis*, *Legionella shakespearei* and *Legionella waltersii*. Conversely, all of the 65 serogroup reference strains were tested by DFA using antisera prepared against strain Gréoux 11 D13^T and against strain Montbéliard A1^T.

Slide agglutination test. Both type strains were tested at the Centers for Disease Control (Atlanta, GA, USA) with

Table 1. Biochemical characteristics of the isolates

w, Weak; + w, weakly positive; v, variable.

Strain	Oxidase	β -Lactamase	Hippurate hydrolysis	Tyrosinase	Gelatinase	Voges–Proskauer
Gréoux 11 D13 ^T	+ w	+ w	+	v	+ (slow)	—
Mulhouse 12 A22	+ w	+	+ w	—	+	—
Mulhouse 12 A23	+ w	+	+ w	—	+	—
Montbéliard A1 ^T	v	+	—	—	+	+
Montbéliard A2	—	+	—	—	+	+
Montbéliard A3	—	+	—	—	+	—
Montbéliard A4	+	+	—	—	+	—
Montbéliard A5	+	+	—	—	+	—

the slide agglutination test (SAT) using antisera representing all species and serogroups of *Legionella*. Antisera prepared against the type strains of both novel species were tested against antigens for all species and serogroups of *Legionella*. Antisera and antigens for the SAT were prepared, tested and absorbed as described previously (Thacker *et al.*, 1985).

Cell wall fatty acids and isoprenoid quinone composition. Cell wall fatty acids and isoprenoid quinones were assayed as described previously (Lambert & Moss, 1989) for strains Montbéliard A1^T, Montbéliard A2, Montbéliard A3, Montbéliard A4 (ubiquinones only), Gréoux 11 D13^T and Mulhouse 12 A22 (fatty acids only) grown on BCYE agar plates for 48–96 h at 37 °C.

Protein profile analysis. The protein electrophoretic patterns of the five isolates from Montbéliard and strain Gréoux 11 D13^T were compared with each other and with other species after SDS-PAGE according to a previously described technique (Bornstein *et al.*, 1989).

Genotypic characterization. DNA was extracted by the boiling water method (Gomez-Lus *et al.*, 1993) and purified with phenol/chloroform/isoamyl alcohol (25:24:1), followed by ethanol precipitation. RAPD analysis was performed with primer SK2 (5'-CGGCGGCGGCGG-3') (Meunier & Grimont, 1993) as described previously (Lo Presti *et al.*, 1997). Amplification of the intergenic 16S–23S ribosomal spacer by PCR was done using primers FGPS1490-72 (5'-TGCGGCTGGATCCCCCTCCTT-3') and FGPL132'-38 (5'-CCGGGTTTCCCCATTCGG-3') as published recently (Riffard *et al.*, 1998). Each RAPD and 16S–23S genotypic profile (both faint and intensive bands were used) was analysed by the TAXOTRON package (Taxolab, Institut Pasteur, Paris, France) using the Dice coefficient (Dice, 1945) and clustering was based on the single linkage method. Profiles for all *Legionella* species and serogroups described and for some additional potentially novel species were available for comparisons.

16S rDNA amplification and sequencing. DNA amplification of the 16S rRNA gene was performed by using oligonucleotide primers L16A1 (5'-AGA GTT GGA TCC TGG CTC AG-3') and L16r1488 (5'-GAC TTC ACC CCA GTC ATG AA-3'), corresponding to positions 8–27 and 1488–1508, respectively, in *Escherichia coli* numbering (Brosius *et al.*, 1978). Sequencing was done by the dideoxy chain termination method (Sanger *et al.*, 1977) under conditions described previously (Lo Presti *et al.*, 1997) and the sequences of strains Gréoux 11 D13^T and Montbéliard A1^T were compared using the gapped BLAST program (Altschul *et al.*, 1997) with previously published sequences (Adeleke *et al.*, 1996; Birtles *et al.*, 1996; Fry *et al.*, 1991; Hookey *et al.*, 1996) and those available in the DDBJ, EMBL and GenBank databases through the Internet (<http://www.ncbi.nlm.nih.gov>). For this study, sequencing of the recently described *L. waltersii* ATCC 51914^T (Benson *et al.*, 1996) and *Legionella pneumophila* subsp. *pascuelli* ATCC 33737^T (Brenner *et al.*, 1988) was performed.

Phylogenetic analyses. Phylogenetic analyses were performed with the Wisconsin package version 10.0 of the Genetics Computer Group (GCG, Madison, WI, USA). The 16S rDNA gene sequences were first aligned using the PILEUP program and the resulting multi-sequence alignment was subsequently manually corrected. A total of 1304 nt ranging from positions 34 to 1338, according to *E. coli* numbering (Brosius *et al.*, 1978), was included in this study. Kimura's two-parameter distance matrices and the resulting unrooted tree were done using DISTANCE and GROWTREE programs.

DNA studies. DNA was extracted and purified as previously described (Brenner *et al.*, 1982) and the native DNA was labelled by nick-translation with ³H-labelled nucleotides (Pharmacia Biotech). DNA–DNA hybridization with all species (Table 3) was performed using the standard S1-nuclease method (Grimont *et al.*, 1980). The thermal stability of heterologous hybrids was calculated by a standard method (Crosa *et al.*, 1973).

The DNA G+C content of our strains was determined by HPLC of nucleotides after hydrolysis of the DNA by P1 nuclease (Kaneko *et al.*, 1986) and also spectrophotometrically by the thermal denaturation method (Marmur & Doty, 1962).

RESULTS

Isolation, growth and biochemical characterization

Strain Gréoux 11 D13^T was isolated from an acid-treated water sample from a shower in a thermal spa in the city of Gréoux, France after 3 d growth on BAB media. The same water sample also yielded *L. pneumophila* (undetermined serogroup 8 or 10), *Legionella anisa*, *Legionella rubrilucens* and *Legionella londinensis*. Strains Mulhouse 12 A22 and Mulhouse 12 A23 were isolated from a water sample obtained from a potash mine located near Mulhouse in France. Strains Montbéliard A1^T to Montbéliard A5 were isolated from GVC media after 10 d growth from an acid-treated water sample from a calorifier in a reanimation unit of a hospital in Montbéliard, France.

All of these isolates grew on BCYE α and GVC media; however, the strains from Montbéliard grew very slowly. None of the isolates grew on blood agar or on BCYE α lacking supplemented L-cysteine. They were non-spore-forming, Gram-negative rods with a single polar flagellum. Biochemical tests were positive for the presence of catalase. These isolates gave negative reactions for urease, carbohydrate fermentation and nitrate reduction. Other biochemical characteristics are shown in Table 1. Autofluorescence under UV light was absent for all isolates; however, a yellow-green fluorescence of the growth medium was produced around the colonies. Colonies were greenish on DGVP medium.

Table 2. Ubiquinone contents of the isolates

For the quinones, the figure indicates the number of isoprene units, e.g. Q-9 indicates that the quinone contains 9 isoprene units. Relative amounts of ubiquinones are shown, with the major component designated by a score of 4. —, None detected; TR, trace amount.

Strain	Q-9	Q-10	Q-11	Q-12	Q-13	Q-14
Gréoux 11 D13 ^T	—	—	1.3	4	0.4	—
Montbéliard A1 ^T	0.2	0.1	0.5	4	1.5	—
Montbéliard A2	0.2	0.1	0.5	4	1.5	TR
Montbéliard A3	0.2	0.2	0.6	4	1.4	—
Montbéliard A4	0.2	0.5	0.9	4	1.6	—

Fig. 1. Unrooted 16S rDNA phylogenetic tree. The GenBank/EMBL accession numbers are given in parentheses. *L. p.* subsp. *pne.*, *Legionella pneumophila* subsp. *pneumophila*; *L. p.* subsp. *pas.*, *Legionella pneumophila* subsp. *pascallei*; *L. p.* subsp. *fra.*, *Legionella pneumophila* subsp. *fraseri*; ser., serogroup.

DFA and SAT

The five strains from Montbéliard gave negative reactions with all 65 heterogeneous antisera tested. The same five isolates reacted with a level of 4+ when tested with an antiserum prepared against strain Montbéliard A1^T. This antiserum was negative when tested against all *Legionella* species and serogroups. In the SAT test, the type strain was negative with specific antisera of each *Legionella* species and serogroups. Antiserum prepared against the type strain gave reactions of 4+ with the homologous strain and was negative when tested against all *Legionella* species and serogroup type strains. Strain Gréoux 11 D13^T first reacted with a level of + to 2+ against *L. anisa* unabsorbed antiserum; however, this reaction was not reproducible. Antiserum developed against the Gréoux 11 D13^T isolate gave a fluorescence of 4+ with the homologous strain and with the two isolates from Mulhouse. No reactions were noted against any *Legionella* species or serogroups. In the SAT test, strain Gréoux 11 D13^T was negative with antisera for all *Legionella* species and serogroups. Antiserum prepared against Gréoux 11 D13^T was negative against all *Legionella* species and serogroups.

Cellular fatty acids and ubiquinone composition

Non-hydroxy fatty acids comprised more than 90 % of the total fatty acid composition for each strain tested. Three strains from Montbéliard contained *cis*-9-hexadecanoate (16:1) as their major fatty acid (31 %), followed by hexadecanoate (16:0) which accounted for 20 %, 18:1 (15 %), octadecanoate (18:0) for 11 % and 14-methylhexadecanoate (a17:0) for 9 %. Other fatty acids were present, but each in quantities less than 8 %. Strains Gréoux 11 D13^T and Mulhouse 12 A22 were also characterized by a large quantity of *cis*-9-hexadecanoate, accounting for 33 % of the total fatty acids. These strains also contained appreciable amounts of 14-methylpentadecanoic acid (i16:0) (13 %), followed by 16:0 and a17:0 (9 %) and minor fatty acids, each accounting for 8 %. Isolates from Montbéliard and strain Gréoux 11 D13^T had ubiquinone profiles dominated by the presence of quinone Q-12 (Table 2).

SDS-PAGE analysis

Protein profiles obtained from strains Montbéliard A1^T, Montbéliard A2 and Montbéliard A3 were 95–100 % homologous. Protein profiles of Montbéliard A4 and Montbéliard A5 were 100 % homologous, but only 74–79 % homologous to other Montbéliard strains. However, homology of the ‘Montbéliard’ cluster was less than 70 % with all known *Legionella* species. The protein profiles of strain Gréoux 11 D13^T were also different from those of all known *Legionella* species (less than 60 % homology).

RAPD analysis

The RAPD profiles obtained for strains belonging to the ‘Montbéliard’ cluster were identical. The two strains from Mulhouse gave a profile identical to that of Gréoux 11 D13^T. The RAPD patterns of these ‘Gréoux’ and ‘Montbéliard’ clusters were different from those obtained for all the other *Legionella* species tested. These species were named *Legionella* sp. 1 and sp. 3, respectively, in a previous publication (Lo Presti *et al.*, 1998).

16S–23S intergenic spacer region PCR analysis

The patterns obtained for strains belonging to the ‘Gréoux’ and ‘Montbéliard’ clusters were unique and could be easily differentiated from any other profile generated by the known *Legionella* species. These two clusters were previously named *Legionella* sp. Type 1 and Type 13, respectively (Riffard *et al.*, 1998).

16S rRNA gene analysis and phylogenetic studies

The comparison of 1430 bp (gaps excluded from the analysis) of the aligned 16S rRNA gene sequences of the strains Gréoux 11 D13^T and Montbéliard A1^T showed they were 97.83 % identical (31 different nucleotides). The nearest species was *L. micdadei* (GenBank no. AF227162) with identities of 96.88 % (1408 nt compared with the sequence for strain Gréoux 11 D13^T) and 95.62 % (1437 nt compared with the sequence for strain Montbéliard A1^T). The two sequences were less related to each of the 16S rDNA sequences of the described *Legionella* species (LLAP strains included). Phylogenetic relationships obtained from these data are shown in Fig. 1.

DNA hybridization

As shown in Table 3, DNA–DNA hybridization studies of strain Gréoux 11 D13^T revealed a high level of reassociation with DNA from isolates Mulhouse 12 A22 and Mulhouse 12 A23, with more than 90 % relatedness in both optimal and stringent reactions. The nearest species was represented by those of the ‘Montbéliard’ cluster with only 24 % relatedness. The four strains from Montbéliard were also more than 95 % inter-related. Again, the nearest species was *L. micdadei* with 22 and 15 % relatedness with strains Gréoux 11 D13^T and Montbéliard A1^T, respectively (see Table 3). The two clusters (‘Montbéliard’ and ‘Gréoux’) exhibited 12 % or less relatedness to all other *Legionella* species.

DNA base composition

The G+C content of strain Gréoux 11 D13^T was 33 mol % by each of the two methods used. Montbéliard A1^T strain had a G+C content of

Table 3. DNA relatedness of strains Gréoux 11 D13^T and Montbéliard A1^T with *Legionella* spp.

Source of unlabelled DNA	Relatedness (%) to labelled DNA from:					
	Gréoux 11 D13 ^T			Montbéliard A1 ^T		
	60 °C*	70 °C*	60 °C†	60 °C*	70 °C*	60 °C
Gréoux 11 D13 ^T (= ATCC 700509 ^T)‡	100	100	100	24	ND	ND
Mulhouse 12 A22 (= ATCC 700758)‡	100	93	ND	ND	ND	ND
Mulhouse 12 A23 (= ATCC 700759)‡	100	92	ND	ND	ND	ND
Montbéliard A1 ^T (= ATCC 700512 ^T)§	24	ND	26	100	100	100
Montbéliard A2 (= ATCC 700760)§	ND	ND	ND	100	100	ND
Montbéliard A3§	ND	ND	ND	100	100	ND
Montbéliard A4§	ND	ND	ND	95	90	ND
<i>Legionella adelaidensis</i> ATCC 49625 ^T	5	ND	ND	2	ND	ND
<i>Legionella anisa</i> ATCC 35292 ^T	5	ND	ND	7	ND	ND
<i>Legionella birminghamensis</i> ATCC 43702 ^T	3	ND	5	10	ND	7
<i>Legionella</i> (<i>Fluoribacter</i>) <i>bozemanii</i> sg.1 ATCC 33217 ^T	5	ND	8	9	ND	8
<i>Legionella brunensis</i> ATCC 43878 ^T	8	ND	6	9	ND	9
<i>Legionella cherrii</i> ATCC 35252 ^T	4	ND	ND	9	ND	ND
<i>Legionella cincinnatiensis</i> ATCC 43753 ^T	6	ND	5	6	ND	7
' <i>Legionella donaldsonii</i> ' strain LC 878	2	ND	ND	11	ND	ND
<i>Legionella</i> (<i>Fluoribacter</i>) <i>dumoffii</i> ATCC 33279 ^T	4	ND	4	8	ND	7
<i>Legionella erythra</i> serogroup 1 ATCC 35303 ^T	8	ND	5	2	ND	9
<i>Legionella fairfieldensis</i> ATCC 49588 ^T	1	ND	6	5	ND	8
<i>Legionella feeleei</i> serogroup 1 ATCC 35072 ^T	1	ND	5	6	ND	9
<i>Legionella geestiana</i> ATCC 49504 ^T	2	ND	4	3	ND	5
<i>Legionella genomospecies</i> 1 (= ATCC 51913)	8	ND	5	9	ND	5
<i>Legionella</i> (<i>Fluoribacter</i>) <i>gormanii</i> ATCC 33297 ^T	5	ND	5	5	ND	6
<i>Legionella gratiana</i> ATCC 49413 ^T	2	ND	7	5	ND	7
<i>Legionella hackeliae</i> serogroup 1 ATCC 35250 ^T	7	ND	6	7	ND	9
<i>Legionella israelensis</i> ATCC 43119 ^T	5	ND	2	7	ND	4
<i>Legionella jamestowniensis</i> ATCC 35298 ^T	7	ND	6	7	ND	8
<i>Legionella jordanis</i> ATCC 33623 ^T	6	ND	4	4	ND	7
<i>Legionella lansingensis</i> ATCC 49751 ^T	10	ND	8	5	ND	10
<i>Legionella londiniensis</i> ATCC 49505 ^T	0	ND	7	6	ND	6
<i>Legionella longbeachae</i> serogroup 1 ATCC 33462 ^T	8	ND	4	8	ND	7
<i>Legionella lytica</i>	ND	ND	ND	ND	ND	ND
<i>Legionella</i> (<i>Tatlockia</i>) <i>maceachernii</i> ATCC 35300 ^T	3	ND	3	6	ND	9
<i>Legionella</i> (<i>Tatlockia</i>) <i>micdadei</i> ATCC 33218 ^T	22	ND	20	6	ND	15
<i>Legionella moravica</i> ATCC 43877 ^T	5	ND	5	6	ND	7
<i>Legionella nautarum</i> ATCC 49506 ^T	5	ND	6	7	ND	9
<i>Legionella oakridgensis</i> ATCC 33761 ^T	3	ND	4	4	ND	ND
<i>Legionella parisiensis</i> ATCC 35299 ^T	5	ND	4	5	ND	6
<i>Legionella pneumophila</i> subsp. <i>pneumophila</i> ATCC 33152 ^T	3	ND	4	3	ND	6
<i>Legionella quateirensis</i> ATCC 49507 ^T	4	ND	6	6	ND	6
<i>Legionella quinlivanii</i> serogroup 1 ATCC 43830 ^T	8	ND	6	8	ND	8
<i>Legionella rubrilucens</i> ATCC 35304 ^T	4	ND	ND	ND	ND	ND
<i>Legionella sainthelensi</i> serogroup 1 ATCC 35248 ^T	12	ND	3	7	ND	7
<i>Legionella santicrucis</i> ATCC 35301 ^T	3	ND	ND	ND	ND	ND
<i>Legionella shakespearei</i> ATCC 49655 ^T	2	ND	5	ND	ND	6
<i>Legionella spiritensis</i> serogroup 1 ATCC 35249 ^T	1	ND	2	ND	ND	6
<i>Legionella steigerwaltii</i> ATCC 35302 ^T	0	ND	ND	ND	ND	ND
<i>Legionella taurinensis</i> ATCC 700508 ^T	0	ND	ND	0	ND	ND
<i>Legionella tucsonensis</i> ATCC 49180 ^T	2	ND	6	ND	ND	8
<i>Legionella wadsworthii</i> ATCC 33877 ^T	1	ND	1	ND	ND	6
<i>Legionella waltersii</i> ATCC 51914 ^T	0	ND	5	ND	ND	5
<i>Legionella worsleiensis</i> ATCC 49508 ^T	5	ND	5	ND	ND	6
Strain IB V no. 2 (= ATCC 700511)	1	ND	5	ND	ND	6

ND, Not done.

* Reactions were done at least in duplicate at 60 °C. Relatedness by S1-nuclease method. Standard deviation was less than 2%. The maximum deviation between two determinations was 5%.

† Relatedness by hydroxyapatite method. Reactions were done in duplicate with mean and maximum deviations of 1.4 and 4.0%, respectively.

‡ Strains of the cluster 'Gréoux'.

§ Strains of the cluster 'Montbéliard'.

38 mol %, whereas strain Montbéliard A4 had a G + C content of 37 mol %.

DISCUSSION

The health risks associated with the presence of *Legionella* isolates in potable and non-potable water available to the public frequently results in a survey of these water systems for *Legionella*. During such investigations, several atypical isolates were recovered. On the basis of cultural criteria, these strains appeared to belong to the genus *Legionella*, but remained untypable by routine techniques.

Among these techniques, biochemical characteristics were typical of the majority of legionellae in that these strains were catalase-positive, but were unable to reduce nitrate to nitrite. They were also negative for carbohydrate fermentation and presence of urease. Two strains from Montbéliard had an unusual biochemical reaction in that they were positive for the Voges-Proskauer test, which is indicative of a possible butylene glycol type of fermentation in these strains.

Except for a weak positive reaction of strain Gréoux 11 D13^T with an unabsorbed antiserum prepared against *L. anisa*, none of the isolates reacted with any *Legionella* species or serogroup-specific antisera. Antisera made against these atypical strains revealed specific serotyping reactions. This absence of identification to the species or serogroup level on the basis of serotyping led us to pursue the identification of these putative *Legionella* isolates by determining their fatty acid composition. The results completely fit the criteria of the genus *Legionella* since their cellular composition is predominantly branch-chained fatty acids (Wilkinson *et al.*, 1990). With their fatty acid composition, both clusters were placed in the C16 group (Lambert & Moss, 1989). The isoprenoid quinone composition of the 'Gréoux' and 'Montbéliard' clusters was also typical of *Legionella* species, with long side chains containing 10 or more isoprene units. This composition placed these two clusters in ubiquinone group A of Lambert & Moss (1989). All these methods (cultural and biochemical characteristics, fatty acid and ubiquinone composition) are useful for ascribing strains to *Legionella* at the genus level, but cannot be used to determine whether they represent novel species or serotypes of existing species.

Previous genotyping analysis by RAPD (Lo Presti *et al.*, 1998) and 16S–23S intergenic spacer region PCR (Riffard *et al.*, 1998) did not resolve their identification in that no good correlations with existing species were found. The two clusters were represented by a unique profile revealing that they were potentially novel species. Moreover, these results correlated well with those obtained by SDS-PAGE analysis and serotyping. Except for the problem of serotypic cross-reaction, these four methods confirmed their usefulness in speciating strains.

When comparing the 16S rDNA sequences of strains Gréoux 11 D13^T and Montbéliard A1^T with each *Legionella* and LLAP 16S rDNA sequence, the highest homology (97.83 %) was found between the two new clusters, whereas the other species (including LLAP strains) were less than 97 % related. The phylogenetic study supported this relationship by clustering them in the same branch of the tree (Fig. 1). It is generally accepted that strains with 16S rRNA gene homology under 97 % are members of different species (Stackebrandt & Goebel, 1994). The level of homology for clusters 'Gréoux' and 'Montbéliard' was less than that necessary to confirm their status as one or two potentially novel species. In previous publications, it has been shown that, in the legionellae, two different species can reach as high as 99.4 % 16S rRNA gene homology (Hookey *et al.*, 1996; Lo Presti *et al.*, 1999). The high level of similarity between the small subunit rDNA gene of the two clusters clearly indicated that DNA pairing studies needed to be performed firstly between them, secondly with *L. micdadei* and then with other *Legionella* species. If these DNA pairing studies revealed them as novel genomic species, it is the presence or absence of specific phenotype that should be the deciding factor about whether to name them.

DNA hybridization studies yielded results similar to those from 16S rDNA comparisons, showing that these two clusters were related but distinct from any other *Legionella* species. These experiments revealed that the two groups completely fitted the genetic definition of a species (Wayne *et al.*, 1987). The two clusters were each homogeneous, with more than 90 % DNA relatedness between the strains tested (Table 3). These two groups showed relatedness for each other, but the DNA–DNA relatedness was only 24 % at optimal reassociation criteria. It is interesting to note that one of their nearest 16S rDNA phylogenetic branches, containing *L. micdadei* and *L. maceachernii*, was confirmed by DNA–DNA hybridization results (15–22 % relatedness), indicating that they are their closest relatives. However, these latter species are easily differentiated from our atypical strains by serotyping and colour of the colonies on DGVP media. The dark blue colour that can be obtained for colonies grown on this media is still restricted to the species *L. micdadei* and *L. maceachernii* (Vickers *et al.*, 1987).

A large number of *Legionella* species has been described on the basis of a single strain description (Brenner *et al.*, 1985; Bornstein *et al.*, 1989; Benson *et al.*, 1996). As legionellae are of medical importance, the description of a novel species, although a unique strain, can help in the identification of legionellosis due to an atypical isolate as shown recently for *Legionella parisiensis* (Lo Presti *et al.*, 1997). In our case, the cluster 'Gréoux' has been characterized with strains isolated from two different locations. Each isolate of this cluster has strictly identical phenotypic characteristics and has been shown by genotypic, 16S rDNA and DNA studies to belong to a unique species. In the case of the cluster 'Montbéliard', the five isolates were

recovered from only one location. The slight phenotypic differences in biochemical characteristics and protein profiles noted for isolates Montbéliard A4 and A5 compared to the type strain revealed that the five selected isolates belonged to at least two different strains. However, DNA studies clearly demonstrated that they belonged to the same species with more than 95% DNA homology. These two novel species can be phenotypically identified by serotyping, a criterion usually required for describing novel *Legionella* species (Benson *et al.*, 1996; Ursing *et al.*, 1995). The sum total of our results confirms the 'Montbéliard' and 'Gréoux' clusters as novel *Legionella* species (*Legionella beliardensis* sp. nov. and *Legionella gresilensis* sp. nov., respectively).

While this manuscript was being edited, three LLAP strains cited in this study were described as three novel species by Adeleke *et al.* (2001): *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. The numbering of our novel species is made according to Adeleke *et al.* (2001).

Description of *Legionella beliardensis* sp. nov.

Legionella beliardensis (be.li.ar.den'sis. M.L. adj. *beliardensis* pertaining to Montbéliard, France, whose classical Latin name was Montem Beliardae).

Gram-negative rod with a single polar flagellum. Positive for catalase, gelatinase and presence of β -lactamase. Negative for urease, nitrate reduction, acid production from D-glucose, browning of tyrosine agar and hippurate hydrolysis. Some strains are positive for the Voges-Proskauer test. DFA and SAT results exhibit no serological cross-reaction with any other *Legionella* species. Major ubiquinone is Q-12. All strains were isolated from water samples from a single French city, Montbéliard. The type strain is Montbéliard A1^T (= ATCC 700512^T = CIP 106632^T), isolated from a calorifier in a hospital in Montbéliard (France). It has a G+C content of 38 mol%. Strains Montbéliard A2 (= ATCC 700760), Montbéliard A3, Montbéliard A4 and Montbéliard A5 are additional strains of this species.

Description of *Legionella gresilensis* sp. nov.

Legionella gresilensis (gre.si.len'sis. M.L. adj. *gresilensis* pertaining to Gréoux, a French spa city, whose classical Latin name was Gresilium).

Gram-negative rod with a single polar flagellum. Positive for catalase, oxidase, gelatinase, hippurate hydrolysis and presence of β -lactamase. Negative for urease, nitrate reduction, acid production from D-glucose and browning of tyrosine agar. DFA and SAT results indicate no serological cross-reactions with any *Legionella* species. Major ubiquinone is Q-12. All

strains were isolated from water samples in France. The type strain is Gréoux 11 D13^T (= ATCC 700509^T = CIP 106631^T), isolated from a thermal spa water in the city of Gréoux (France). It has a G+C content of 33 mol%. Strains Mulhouse 12 A22 (= ATCC 700758) and Mulhouse 12 A23 (= ATCC 700759) are additional strains of *Legionella gresilensis*.

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