The use of 16S ribosomal RNA analyses to investigate the phylogeny of the family *Legionellaceae*

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The 16S ribosomal RNA sequences of *Legionella pneumophila*, *L. erythra*, *L. hackeliae*, *L. spiritensis*, *L. longbeachae*, *L. bozemanii* (*Fluoribacter bozemanii*) and *L. micdadei* (*Tatlockia micdadei*) were determined using reverse transcriptase. The sequences were compared with published sequences for Gram-negative bacteria and phylogenetic trees were constructed. The data confirm previous work which showed that the family *Legionellaceae* forms a monophyletic subgroup within the gamma subdivision of the Proteobacteria. The data show that all of the legionellae studied are highly related (>95%) on the basis of 16S rRNA sequences and do not support the division of the family *Legionellaceae* into three genera.

Introduction

The family *Legionellaceae* was initially proposed for a single genus *Legionella* and species *L. pneumophila*, the causal agent of Legionnaires' disease (Brenner *et al.*, 1979). Subsequently large numbers of new species of *Legionella* have been isolated from clinical and environmental samples (Brenner, 1986; Brenner *et al.*, 1985, 1988). All of these isolates share a common set of phenotypic characteristics that include a non-fermentative metabolism, the requirement for L-cysteine and iron salts for growth, ubiquinones with 10 or more isoprene units, and predominantly branched-chain cellular fatty acids (Karr *et al.*, 1982; Brenner *et al.*, 1984; Brenner, 1986). The identification of legionellae at the species level is more difficult as there are relatively few suitable phenotypic tests (Brenner, 1986; Brenner *et al.*, 1984; Fox *et al.*, 1984, 1990; Vesey *et al.*, 1988). New species are identified by DNA pairing measurements, and this method is the only means by which some species may be distinguished (Brenner, 1986; Brenner *et al.*, 1988). However, DNA pairing values between species within the genus *Legionella* may vary between 0% and 67% under optimum reassociation conditions (Garrity *et al.*, 1980; Brown *et al.*, 1981; Brenner, 1986). The very low DNA hybridization values that are obtained between some species of *Legionella* have been interpreted differently. One group (Garrity *et al.*, 1980; Brown *et al.*, 1981) has advocated that the family *Legionellaceae* be subdivided into three genera. The genus *Fluoribacter* was

proposed for strains of *Legionella bozemanii* which exhibit blue-white autofluorescence and show less than 14% DNA relatedness to *L. pneumophila* (Garrity *et al.*, 1980; Brown *et al.*, 1981). A further genus, *Tatlockia*, was proposed for *L. micdadei* (Hébert *et al.*, 1980b), which shows less than 5% DNA relatedness to *L. pneumophila* (Garrity *et al.*, 1980; Brown *et al.*, 1981). In contrast to these proposals, others have argued that all legionellae are sufficiently similar and unusual in phenotype to be classified in a single genus (Brenner *et al.*, 1980, 1984; Hébert *et al.*, 1980a, b; Morris *et al.*, 1980; Brenner, 1986). At present there is no clear agreement as to which position is correct and the same strains appear in the Approved Lists of Bacterial Names under two different names.

The sequence of the genes that code for ribosomal RNA (rRNA) are among the most highly conserved yet identified (Brenner, 1986; Woese, 1987). Consequently they can be used to determine taxonomic relationships between species which show little DNA interrelatedness (Brenner, 1986). The accuracy of phylogenetic inferences from rRNA sequences depends upon the number of bases compared, and to be fully effective at least 1000 bases should be considered for each organism (Murray *et al.*, 1990). Ludwig & Stackebrandt (1983) investigated the phylogeny of *Legionella pneumophila*, *Legionella bozemanii* (*F. bozemanii*) and *Legionella micdadei* (*T. micdadei*) using oligonucleotide cataloguing of 16S rRNA, which recovers about 40% of the 16S rRNA sequence. The results of that study showed that all three

organisms were related and well separated from other Gram-negative bacteria but did not resolve whether there should be one or three genera. Fox & Brown (1989) used reverse transcriptase to sequence short stretches (350–450 bases) of 16S rRNA from three strains of *L. pneumophila* and three of *L. micdadei* (*T. micdadei*). However, they were unable to draw any conclusions about the taxonomic structure of the family *Legionellaceae*. In the current investigation we have attempted to determine the detailed relationships of the family *Legionellaceae* by sequencing long stretches (about 95%) of the 16S rRNA from a representative sample of species including *L. pneumophila*, *L. erythra*, *L. hackeliae*, *L. spiritensis*, *L. longbeachae*, *L. bozemanii* (*F. bozemaniae*) and *L. micdadei* (*T. micdadei*).

Methods

Bacterial strains and cultivation. The test strains (Table 1) were inoculated onto buffered charcoal yeast-extract agar (BCYE) and incubated for 48 h at 37 °C. The cells were removed from the surface of the plates using sterile saline (0.85%, w/v), pelleted by centrifugation at 10000 g, and stored at –70 °C.

Isolation of RNA. The method used was modified from that described by Saunders & Grant (1984). Approximately 2 g wet weight of cells was resuspended in 5 ml of a buffer containing 25% (w/v) sucrose, 50 mM-Tris/HCl (pH 8.0) and 3.3 mg lysozyme ml^{–1}, and incubated for 30 min at 37 °C. The cells were recovered by centrifugation (1400 g), resuspended in 20 ml 10 mM-Tris/HCl (pH 7.5) buffer containing 1% (w/v) SDS, 5 mM-EDTA and 200 µg proteinase K ml^{–1} (Sigma), and incubated for 1 h at 45 °C. The lysate was extracted twice with equilibrated phenol (Maniatis *et al.*, 1982) and then twice with chloroform/isoamyl alcohol (24:1, v/v). The aqueous phases were pooled and adjusted to 0.3 M with respect to sodium acetate (pH 6.0).

Total nucleic acids were precipitated by incubation with 2 vols ethanol at –20 °C. The nucleic acid pellet was resuspended in 20 ml sodium acetate (pH 6.0) and the DNA was sheared by passing the suspension repeatedly through a sterile 23 gauge needle until the viscosity decreased significantly. The suspension was then centrifuged again and the dried pellet resuspended in 0.5 ml TE buffer (10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA) containing 6 mM-MgCl₂. RNAase-free DNAase (37.5 units, Pharmacia LKB) was then added and the mixture incubated for 30 min at 20 °C. The RNA was precipitated with 2 vols ethanol at –20 °C, collected by centrifugation (10000 g) and the dried pellet was resuspended in 50 µl sterile distilled water to which 1.5 ml 3 M-sodium acetate (pH 6.0) was added. After incubation for 2 h at 4 °C the RNA was collected by centrifugation (10000 g) and the pellet washed with 0.1 M-sodium acetate (pH 6.0)/70% (v/v) ethanol prior to redissolving in 100 µl sterile distilled water. The integrity of the rRNA was determined by electrophoresis in an agarose (1%, w/v) mini-gel prepared using TBE buffer (0.1 M-Tris, 83 mM-boric acid, 1 mM-EDTA, pH 8.6) and containing 5 µg ethidium bromide ml^{–1}. The concentration of the RNA solution was adjusted to 3 µg µl^{–1} and stored at –70 °C.

RNA sequencing. Eight primers were routinely used to generate sequence data. The sequences and provenance of primers have been given previously (Embley *et al.*, 1988). The method used for sequencing 16S rRNA was that devised by Lane *et al.* (1985, 1988) with slight modifications as follows. The reverse transcription reaction mixture contained 7 units of AMV reverse transcriptase (Stratagene) and 10 µCi (370 kBq) deoxyadenosine 5'-α-[³⁵S]thiotriphosphate (Du Pont; specific activity 1300 Ci mmol^{–1}, 48.1 TBq mmol^{–1}). The nucleotide mixes were prepared using individual ultrapure nucleotide solutions (Pharmacia LKB). Stock mixes were determined empirically to give about 300–350 bases of readable sequence and comprised: A mix, dATP 20 µM, dCTP dGTP dTTP each at 1.7 mM, ddATP 3.2 µM; C mix, dATP 8.5 µM, dCTP 0.4 mM, dGTP and dTTP each 1.7 mM, ddCTP 32.5 µM; G mix, dATP 8 µM, dCTP and dTTP each 1.6 mM, dGTP 0.4 mM, ddGTP 55.8 µM; T mix, dATP 9 µM, dCTP and dGTP each 1.8 mM, dTTP 0.3 mM, ddTTP 67 µM. The chase mix contained each dNTP at 0.5 mM. The products of the sequencing reactions were separated on 55 cm wedge-shaped (0.2–0.6 mm) 6% (w/v) polyacrylamide denaturing (7 M-urea) gels using an LKB Macrophor system

Table 1. *Legionella* strains included in this study

Species	Strain designation	Source	GenBank no. of rRNA sequence
<i>L. pneumophila</i>	Philadelphia 1	NCTC 11192*	M36023
subsp. <i>pneumophila</i>	Knoxville 1	NCTC 11286	M36024
<i>L. pneumophila</i>	Los Angeles 1	NCTC 11233*	M36025
subsp. <i>fraseri</i>	Dallas 1E	NCTC 11405	M36026
<i>L. erythra</i>	SE 32A C8	NCTC 11977*	M36027
<i>L. hackeliae</i>	Lansing 2	NCTC 11979*	M36028
<i>L. longbeachae</i>	Long Beach 4	NCTC 11477*	M36029
<i>L. spiritensis</i>	Mt St Helens 9	NCTC 11990*	M36030
<i>L. bozemanii</i> (<i>Fluoribacter bozemaniae</i>)	WIGA	NCTC 11368*	M36031
<i>L. micdadei</i> (<i>Tatlockia micdadei</i>)	TATLOCK	NCTC 11371*	M36032

* Denotes type strain.

operated at 50 W per gel and at 60 °C. Prior to loading the samples, and after 10 min pre-electrophoresis, sodium acetate (3 M in TBE buffer) was added to the lower chamber to give a final concentration of 0.33 M (Brown & Lund, 1988). After a further 10 min pre-electrophoresis the samples were loaded and allowed to run until the bromophenol blue tracking dye reached the bottom of the gel (about 4–5 h). Urea was removed by washing (twice, 10 min) in a solution comprising 10% (v/v) methanol, 10% (v/v) acetic acid and 2% (w/v) glycerol, followed by a rinse in distilled water. The gel was dried overnight at 80 °C in a standard oven. Autoradiography was for 24–48 hours using Fuji RX film.

Data analysis. Sequences were aligned and similarity values calculated using the ALIGN program (Wilbur & Lipman, 1983) from the DNASTAR suite of programs. The *Legionella* strains and 13 sequences of Gram-negative bacteria representing the major subgroups of the Proteobacteria were included in these calculations (Dams *et al.*, 1988; Rossau *et al.*, 1988; Stackebrandt *et al.*, 1988; Dewhirst *et al.*, 1989; Dorsch *et al.*, 1989; Weisburg *et al.*, 1989; L. M. Manchester, S. Warwick, W. D. Grant & T. M. Embley, unpublished data). Positions where a nucleotide could not be determined unambiguously (designated by an IUB uncertainty code) were not included in the alignment. Regions of extreme variation were removed to eliminate noise due to multiple mutations at a single position, and to ensure that only homologous positions were compared. Seven long stretches of sequence (about 1350 bases) were compared (*Escherichia coli* positions; Brosius *et al.*, 1978): 2–68, 99–190, 220–840, 846–998, 1004–1023, 1037–1132, 1143–1444. The homology values between the sequences were converted to evolutionary distance or K_{nuc} values (Hori, 1975; Jukes & Cantor, 1969). These values were used to produce phylogenetic trees using the algorithm of Fitch & Margoliash (1967) contained in the PHYLIP 3.22 program (J. Felsenstein, University of Washington). The relationships between the Legionellaceae, *Coxiella burnetii*, *E. coli* and *Wolbachia persica* (as outgroups) were also analysed using the program DNABOOT from the PHYLIP 3.22 program. DNABOOT computes a consensus phylogenetic tree using parsimony and the bootstrap method of sampling sequence data.

Results and Discussion

The sequencing strategy used in the current investigation generated between 1446 and 1474 bases (about 95%) of 16S rRNA sequence for the ten strains investigated. The primary sequence data have been deposited in GenBank and assigned the accession numbers shown in Table 1. Fig. 1 shows the matrix of homology values and corresponding K_{nuc} values obtained from a comparison of approximately 1350 bases of sequence from the legionellae and representatives of the major groupings within the Proteobacteria (Stackebrandt *et al.*, 1988). Fig. 2 is a phylogenetic tree showing the position of the family Legionellaceae. The family forms a subline within the gamma subdivision of the Proteobacteria. This confirms the results of earlier phylogenetic studies which showed the legionellae to be distant relatives of this group (Ludwig & Stackebrandt, 1983; Weisburg *et al.*, 1989). The relationship is supported by the presence of the signature oligonucleotide CUAACUYYG, which is present at position 510 (*E. coli* numbering) in all members of the gamma subgroup (Stackebrandt *et al.*,

1988). The two representatives of the Rickettsiaceae, *Coxiella burnetii* and *Wolbachia persica*, showed the closest relationship to the Legionellaceae. The common evolutionary origin of *L. pneumophila* and *C. burnetii*, the causal agent of Q fever, was previously established by Weisburg *et al.* (1989). In this respect it is interesting that some of the bacteria classified as legionellae were originally described as rickettsia-like organisms because of their biological characteristics (Tatlock, 1944; Bozeman *et al.*, 1968).

Fig. 3 shows the internal relationships within the family Legionellaceae based on an analysis of 1350 bases. The tree shown is rooted using *Wolbachia persica* as an outgroup. The consensus tree obtained using the bootstrap parsimony method had exactly the same topology as this tree. The strains of *Legionella* show at least 95% similarity over the 1350 bases of rRNA sequence compared. These values are higher than the similarities (about 90.6%) based on approximately 400 bases of sequence which were detected between *L. micdadei* (*T. micdadei*) and *L. pneumophila* by Fox & Brown (1989). The discrepancies are due to the fact that the short stretches of sequence which they compared contained a high proportion of the highly variable regions of RNA sequence. In contrast, the data presented here are based upon an analysis of approximately 90% of the molecule. The representatives of *Legionella*, including *L. bozemanii* (*F. bozemaniae*) and *L. micdadei* (*T. micdadei*), form a cluster which is of a similar depth to the branch between *E. coli* and *Hafnia alvei* (Fig. 2). It is, however, much shallower than published values between species of genera such as *Mycobacterium* (Smida *et al.*, 1988), *Pseudomonas* (Johnson & Palleroni, 1989), *Saccharopolyspora* (Bowen *et al.*, 1989) and *Streptomyces* (Witt *et al.*, 1989). Fig. 3 shows that *L. bozemanii* (*F. bozemaniae*) is closely related (97.7%) to *L. longbeachae* and forms a sub-branch of the line leading to the subspecies of *L. pneumophila*. This relationship was recovered irrespective of which reference organism, i.e. *C. burnetii*, *E. coli* or *W. persica*, was used as an outgroup. It was also recovered in the bootstrap parsimony analysis (not shown). The classification of *L. bozemanii* (*F. bozemaniae*) has been the subject of considerable debate (Garrity *et al.*, 1980; Ludwig & Stackebrandt, 1983; Brenner, 1986; Brenner *et al.*, 1988). The name *L. bozemanii* was effectively published for a *Legionella*-like organism isolated from diseased lung tissue and which exhibited blue-white autofluorescence under long-wave UV light (Brenner *et al.*, 1980). This strain showed less than 13% DNA relatedness to *L. pneumophila* (Brenner *et al.*, 1980; Garrity *et al.*, 1980). Subsequently, the new genus *Fluoribacter* was validly proposed for the same strain (Garrity *et al.*, 1980). Both names currently appear in the Approved Lists of Bacterial Names and it has been left to

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	
1. <i>Agrobacterium tumefaciens</i>	0.0615	0.0615	0.2313	0.1993	0.2045	0.2019	0.1993	0.1915	0.2151	0.1812	0.1812	0.1812	0.1812	0.1838	0.1851	0.1787	0.1800	0.1749	0.1863	0.1800	0.1711	0.1863	0.1889	0.2191	0.2313	
2. <i>Rochalimea quintana</i>	94.1	0.0593	0.2245	0.2164	0.2019	0.2111	0.2124	0.2032	0.2191	0.1915	0.1915	0.1915	0.1928	0.1941	0.1915	0.1815	0.1889	0.1762	0.1954	0.1915	0.1889	0.2045	0.2178	0.2151	0.2520	
3. <i>Brucella abortus</i>	94.1	94.3	0.2178	0.2019	0.2032	0.1902	0.2006	0.1993	0.2191	0.2006	0.2006	0.1967	0.2032	0.1863	0.1838	0.2006	0.1800	0.1928	0.1876	0.1825	0.1954	0.2071	0.2124	0.2395		
4. <i>Pseudomonas testosteroni</i>	80.1	80.6	81.1	0.1085	0.1537	0.1980	0.2006	0.1941	0.2204	0.1980	0.1980	0.1954	0.2045	0.2019	0.1915	0.1980	0.1967	0.1954	0.1980	0.1863	0.2124	0.2450	0.2353	0.2464		
5. <i>Pseudomonas cepacia</i>	82.5	81.2	82.3	89.9	0.1196	0.1851	0.1941	0.1889	0.2032	0.1762	0.1762	0.1749	0.1762	0.1736	0.1624	0.1624	0.1686	0.1674	0.1624	0.1599	0.2006	0.2450	0.2085	0.2340		
6. <i>Neisseria gonorrhoeae</i>	82.1	82.3	82.2	86.1	89.6	0.1967	0.1928	0.1967	0.2071	0.1992	0.1992	0.1889	0.2006	0.2032	0.1993	0.2032	0.2006	0.1876	0.2058	0.2019	0.2164	0.2436	0.2151	0.2464		
7. <i>Escherichia coli</i>	82.3	81.6	83.2	82.6	83.6	82.7	0.0337	0.0615	0.1248	0.1587	0.1587	0.1674	0.1649	0.1599	0.1537	0.1624	0.1537	0.1624	0.1674	0.1736	0.1812	0.2204	0.2124	0.2326		
8. <i>Hafnia alvei</i>	82.5	81.5	82.4	82.4	82.9	83.0	96.7	0.0604	0.1178	0.1736	0.1736	0.1749	0.1812	0.1711	0.1724	0.1724	0.1225	0.1749	0.1800	0.1915	0.1736	0.2381	0.2019	0.2464		
9. <i>Proteus vulgaris</i>	83.1	82.2	82.5	82.9	83.3	82.7	94.1	94.2	0.1367	0.1674	0.1674	0.1699	0.1724	0.1686	0.1599	0.1699	0.1562	0.1800	0.1699	0.1736	0.1774	0.2285	0.2151	0.2422		
10. <i>Ramirobacter amylophilus</i>	81.3	81.0	81.0	80.9	82.2	81.9	88.5	89.1	87.5	0.1525	0.1525	0.1537	0.1574	0.1391	0.1416	0.1476	0.1513	0.1537	0.1513	0.1674	0.1649	0.2395	0.2245	0.2436		
11. <i>Legionella pneumophila Philadelphia 1</i>	83.9	83.1	82.4	82.6	84.3	82.5	85.7	84.5	85.0	86.2	100.0	0.0000	0.0070	0.0097	0.0348	0.0358	0.0254	0.0411	0.0265	0.0337	0.1237	0.1611	0.2271	0.2245	0.2506	
12. <i>Legionella pneumophila Knoxville 1</i>	83.9	83.1	82.4	82.6	84.3	82.5	85.7	84.5	85.0	86.2	100.0	0.0070	0.0097	0.0348	0.0358	0.0254	0.0411	0.0265	0.0337	0.1237	0.1611	0.2271	0.2245	0.2506		
13. <i>Legionella pneumophila Los Angeles 1</i>	83.9	83.0	82.7	82.8	84.4	83.3	85.0	84.4	84.8	86.1	99.3	99.3	0.0020	0.0411	0.0369	0.0275	0.0422	0.0296	0.0400	0.1213	0.1587	0.2340	0.2245	0.2661		
14. <i>Legionella pneumophila Dallas 1E</i>	83.7	82.9	82.2	82.1	84.3	82.4	85.2	83.9	84.6	85.8	99.1	99.1	99.8	0.0390	0.0390	0.0285	0.0422	0.0306	0.0401	0.1225	0.1611	0.2395	0.2285	0.2675		
15. <i>Legionella erythra</i>	83.6	83.1	83.5	82.3	84.5	82.2	85.6	84.7	84.9	87.3	96.6	96.6	96.0	96.2	0.0306	0.0379	0.0285	0.0390	0.0379	0.1166	0.1513	0.2286	0.2124	0.2478		
16. <i>Legionella hackeliae</i>	84.1	83.6	83.7	83.1	85.4	82.5	86.1	84.6	85.6	87.1	96.5	96.5	96.4	96.2	97.0	0.0411	0.0285	0.0422	0.0379	0.1154	0.1183	0.2191	0.2058	0.2422		
17. <i>Legionella longbeachae</i>	84.0	83.3	82.4	82.6	85.4	82.2	85.4	84.6	84.8	86.6	97.5	97.5	97.3	97.2	96.3	96.0	0.0422	0.0234	0.0453	0.1225	0.1574	0.2204	0.2138	0.2576		
18. <i>Legionella spiritensis</i>	84.4	84.3	84.0	82.7	84.9	82.4	86.1	88.7	85.9	86.3	96.0	96.0	95.9	95.9	97.2	97.2	95.9	0.0490	0.0432	0.1225	0.1501	0.2258	0.2272	0.2450		
19. <i>Legionella bozemanii</i>	83.5	82.8	83.0	82.1	84.3	83.4	85.4	84.4	84.0	86.1	97.4	97.4	97.1	97.0	96.2	95.9	97.7	95.2	0.0453	0.1225	0.1611	0.2340	0.2178	0.2647		
20. <i>Legionella micdadei</i>	84.0	83.1	83.4	82.6	85.4	82.0	85.0	84.0	84.8	86.3	96.7	96.7	96.1	96.1	96.3	96.3	95.6	95.8	88.7	88.7	88.0	0.1587	0.2217	0.1928	0.2506	
21. <i>Coxiella burnetii</i>	84.7	83.3	83.8	83.5	85.6	82.3	84.5	83.1	84.5	85.0	88.6	88.6	88.8	88.7	89.2	89.3	88.7	88.7	88.7	88.0	85.7	85.4	85.7	0.2218	0.2340	0.2534
22. <i>Molbachia persica</i>	83.5	82.1	82.8	81.5	82.4	81.2	83.9	84.5	84.2	85.2	85.5	85.5	85.7	85.5	86.3	87.0	85.8	86.4	85.5	85.4	85.7	80.8	80.8	0.2032	0.2151	
23. <i>Desulfovibrio desulfuricans</i>	83.3	81.1	81.9	79.1	79.1	79.2	80.9	79.6	80.3	79.5	80.4	80.4	79.9	79.5	80.3	81.0	80.9	80.5	79.9	79.8	80.8	80.8	80.8	0.2032	0.2151	
24. <i>Mycobacterium xanthus</i>	81.0	81.3	81.5	79.8	81.8	81.3	82.2	82.3	81.3	80.6	80.6	80.6	80.6	80.6	80.3	81.5	82.0	81.4	80.4	81.1	81.2	83.0	79.9	82.2	0.2299	
25. <i>Mollicella succinogenes</i>	80.1	78.6	79.5	79.0	79.9	79.0	81.5	79.0	79.3	79.2	78.7	78.7	77.6	77.5	78.9	79.3	78.2	79.1	77.7	79.6	78.7	78.5	81.3	80.2		

Fig. 1. Homology values (lower left) and K_{nuc} values (upper right) for 1350 bases of 16S rRNA from representative Proteobacteria and members of the family *Legionellaceae*.

Fig. 2. Unrooted phylogenetic tree showing the position of the family *Legionellaceae* within the confines of the gamma subdivision of the Proteobacteria. Genera are abbreviated as follows: A., *Agrobacterium*; B., *Brucella*; Cx., *Coxiella*; D., *Desulfovibrio*; E., *Escherichia*; H., *Hafnia*; M., *Myxococcus*; N., *Neisseria*; P., *Proteus*; Ps., *Pseudomonas*; R., *Ruminobacter*; Ro., *Rochalimaea*; Wolb., *Wolbachia*; Woli., *Wolinella*.

Fig. 3. Phylogenetic tree showing the relationships within the family *Legionellaceae*. The tree is rooted by reference to *Wolbachia persica*.

individuals to decide which name they wish to use. A critical argument in the proposal for *Fluoribacter bozemanii* (Garrity *et al.*, 1980) was a reference to published DNA data for relationships between genera in the family *Enterobacteriaceae* (Brenner, 1973). This suggested that members of the same genus should show at least 25% DNA relatedness under optimum condi-

tions. However, a number of workers have argued that the genetic relationships between members of the *Enterobacteriaceae* are uniquely high among eubacterial genera, and are thus an inappropriate model for defining relationships in other taxa (Ludwig & Stackebrandt, 1983; Brenner, 1986; Brenner *et al.*, 1988). Indeed there are many examples of good phenotypic species which

show low levels of DNA relatedness in genera such as *Streptomyces* (Mordarski *et al.*, 1986) and *Staphylococcus* (Freney *et al.*, 1988). In a further publication, Garrity and coworkers (Brown *et al.*, 1981) transferred two additional species of autofluorescing legionellae to the genus *Fluoribacter*, as *F. dumoffii* and *F. gormanii*. With the inclusion of these strains the level of DNA relatedness within the genus *Fluoribacter* dropped to as little as 4% between some strains (Brown *et al.*, 1981). In contrast, DNA relatedness between some fluoribacters and the non-autofluorescing *L. longbeachae* and *L. wadsworthii* became as high as 17% and 30%, respectively (Brenner *et al.*, 1985; Brenner, 1986). The results of DNA pairing studies therefore fail to separate *Fluoribacter* from non-autofluorescing strains of *Legionella* (Brenner *et al.*, 1985). In our investigation, *L. bozemanii* (*F. bozemaniae*) clearly falls within the phylogenetic radiation defined by non-autofluorescent species of *Legionella*.

The genus *Tatlockia* was proposed (Brown *et al.*, 1981) for a strain previously classified as *Legionella micdadei* (Hébert *et al.*, 1980b). The main rationale behind this proposal was the inability to detect any DNA homology between this strain and *L. pneumophila* (Brown *et al.*, 1981). Subsequent work has shown that *L. micdadei* (*T. micdadei*) shows moderate levels of relatedness to *L. maceachernii* (23%) and *L. spiritensis* (16%) (Brenner *et al.*, 1985). In the current investigation *L. micdadei* (*T. micdadei*) showed 95–96% similarity in rRNA sequence (over 1350 bases) to strains of *L. pneumophila*, *L. spiritensis*, *L. erythra*, *L. hackeliae* and *L. bozemanii* (*F. bozemaniae*) (Fig. 1). In addition, *L. micdadei* was found to lie within the confines of the radiation defined by *Legionella* in all of the phylogenetic trees constructed from our data.

The strains of *L. pneumophila* were recovered as two closely related (>99% similarity) clusters which represented the two subspecies *pneumophila* and *fraseri*. The two strains of *L. pneumophila* subsp. *pneumophila* were indistinguishable for the entire 1470 bases of 16S rRNA sequence considered. The two strains of *L. pneumophila* subsp. *fraseri* differed at only two positions over 1470 bases. The two subspecies could be differentiated by seven base changes and a single deletion. All of the *L. pneumophila* isolates had a characteristic oligonucleotide UUAUAUGAAAAUAA at position 837 (*E. coli* numbering), which served to differentiate them from the other legionellae. *L. erythra*, *L. spiritensis* and *L. hackeliae* defined the periphery of the family *Legionellaceae* in all of the phylogenetic trees. However, the juxtaposition of the three species showed slight variation depending upon the choice of outgroup. Very little can therefore be concluded concerning their specific relationships.

It has recently been suggested that the integrated use

of phylogenetic and phenotypic characters is necessary for the delineation of bacterial taxa at all levels (Murray *et al.*, 1990). *L. bozemanii* (*F. bozemaniae*) and *L. micdadei* (*T. micdadei*) can be distinguished from other species of *Legionella* using detailed analysis of carbohydrate composition (Fox *et al.*, 1984, 1990), and quantitative fatty acid and ubiquinone composition (Karr *et al.*, 1982; Lambert & Moss, 1989). However, the spectrum of variation of these features is of the same magnitude as found between other species of *Legionella* and is therefore insufficient to justify separate genera for these strains (Brenner *et al.*, 1984; Lambert & Moss, 1989; Fox *et al.*, 1990). The major evidence for the separation of the *Legionellaceae* into different genera stems from the interpretation of the low DNA pairing values which are obtained between some species (Garrity *et al.*, 1980; Brown *et al.*, 1981; Brenner *et al.*, 1985). At the moment these cannot be consistently interpreted to recognize phenotypically coherent groups. In contrast to the low DNA pairing values published for legionellae, all of the species examined in the present study were found to be highly related at the rRNA sequence level. Furthermore, *L. bozemanii* (*F. bozemaniae*) and *L. micdadei* (*T. micdadei*) clearly fall within the radiation defined by *Legionella*. It is therefore phylogenetically invalid to place these species in separate genera. In the absence of compelling phenotypic differences (Brenner, 1986; Murray *et al.*, 1990) we conclude that all of the species examined in the current investigation should be regarded as members of the genus *Legionella* (Brenner *et al.*, 1980; Hébert *et al.*, 1980b; Morris *et al.*, 1980).

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References

- BOWEN, T., STACKEBRANDT, E., DORSCH, M. & EMBLEY, T. M. (1989). The phylogeny of *Amycolata autotrophica*, *Kibdelosporangium aridum* and *Saccharothrix australiensis*. *Journal of General Microbiology* **135**, 2529–2536.
- BOZEMAN, F. M., HUMPHRIES, J. W. & CAMPBELL, J. M. (1968). A new group of rickettsia-like agents recovered from guinea pigs. *Acta Virologia* **12**, 87–93.
- BRENNER, D. J. (1973). Deoxyribonucleic acid reassociation in the taxonomy of enteric bacteria. *International Journal of Systematic Bacteriology* **23**, 298–307.
- BRENNER, D. J. (1986). Classification of Legionellaceae: current status and remaining questions. *Israel Journal of Medical Sciences* **22**, 620–632.
- BRENNER, D. J., STEIGERWALT, A. G. & MCDADE, J. E. (1979). Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family *Legionellaceae*, familia nova. *Annals of Internal Medicine* **90**, 656–658.
- BRENNER, D. J., STEIGERWALT, A. G., GORMAN, G. W., WEAVER, R. E., FEELEY, J. C., CORDES, L. G., WILKINSON, H. W., PATTON, C., THOMASON, B. M. & SASSEVILLE, K. R. L. (1980). *Legionella*

- bozemanii* sp. nov. and *Legionella dumoffii* sp. nov.: classification of two additional species of *Legionella* associated with human pneumonia. *Current Microbiology* **4**, 111–116.
- BRENNER, D. J., FEELEY, J. C. & WEAVER, R. E. (1984). Family Legionellaceae. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 279–288. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- BRENNER, D. J., STEIGERWALT, A. G., GORMAN, G. W., WILKINSON, H. W., BIBB, W. F., HACKEL, M., TYNDALL, R. L., CAMPBELL, J., FEELEY, J. C., THACKER, W. L., SKALIY, P., MARTIN, W. T., BRAKE, B. J., FIELDS, B. S., MCEACHERN, H. V. & CORCORAN, L. K. (1985). Ten new species of *Legionella*. *International Journal of Systematic Bacteriology* **35**, 50–59.
- BRENNER, D. J., STEIGERWALT, A. G., EPPEL, P., BIBB, W. F., MCKINNEY, R. M., STARNES, R. W., COLVILLE, J. M., SELANDER, R. K., EDELSTEIN, P. H. & MOSS, C. W. (1988). *Legionella pneumophila* serogroup Lansing 3 isolated from a patient with fatal pneumonia, and descriptions of *L. pneumophila* subsp. *pneumophila* subsp. nov., *L. pneumophila* subsp. *fraseri* subsp. nov., and *L. pneumophila* subsp. *pascuilei* subsp. nov. *Journal of Clinical Microbiology* **26**, 1695–1703.
- BROSIOUS, J., PALMER, M. L., KENNEDY, P. J. & NOLLER, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 4801–4805.
- BROWN, A., GARRITY, G. M. & VICKERS, R. M. (1981). *Fluoribacter dumoffii* (Brenner *et al.*) comb. nov. and *Fluoribacter gormanii* (Morris *et al.*) comb. nov. *International Journal of Systematic Bacteriology* **31**, 111–115.
- BROWN, N. L. & LUND, P. A. (1988). DNA sequencing. *Methods in Microbiology* **21**, 253–304.
- DAMS, E., HENDRIKS, L., VAN DE PEER, Y., NEEFS, J.-M., SMITS, G., VANDENBEMPT, I. & DE WACHTER, R. (1988). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research* **16**, Supplement: r87–r175.
- DEWHIRST, F. E., PASTER, B. J. & BRIGHT, P. L. (1989). *Chromobacterium*, *Eikenella*, *Kingella*, *Neisseria*, *Simonsiella*, and *Vitreoscilla* species comprise a major branch of the beta group Proteobacteria by 16S ribosomal ribonucleic acid sequence comparison: transfer of *Eikenella* and *Simonsiella* to the family *Neisseriaceae* (emend.). *International Journal of Systematic Bacteriology* **39**, 258–266.
- DORSCH, M., MORENO, E. & STACKEBRANDT, E. (1989). Nucleotide sequence of the 16S rRNA from *Brucella abortus*. *Nucleic Acids Research* **17**, 1765.
- EMBLEY, T. M., SMIDA, J. & STACKEBRANDT, E. (1988). Reverse transcriptase sequencing of 16S ribosomal RNA from *Faenia rectivirgula*, *Pseudonocardia thermophila* and *Saccharopolyspora hirsuta*, three wall type IV actinomycetes which lack mycolic acids. *Journal of General Microbiology* **134**, 961–966.
- FITCH, W. M. & MARGOLASH, E. (1967). Construction of phylogenetic trees: a method based on mutational distances as estimated from cytochrome *c* sequences is of general applicability. *Science* **155**, 279–284.
- FOX, K. F. & BROWN, A. (1989). Partial sequence analysis of the 16S-rRNA of legionellae: taxonomic implications. *Systematic and Applied Microbiology* **11**, 135–139.
- FOX, A., LAU, P. Y., BROWN, A., MORGAN, S. L., ZHU, Z.-T., LENA, M. & WALLA, M. D. (1984). Capillary gas chromatographic analysis of carbohydrates of *Legionella pneumophila* and other members of the family Legionellaceae. *Journal of Clinical Microbiology* **19**, 326–332.
- FOX, A., ROGERS, J. C., FOX, K. F., SCHNITZER, G., MORGAN, S. L., BROWN, A. & AONO, R. (1990). Chemotaxonomic differentiation of legionellae by detection and characterisation of amino-dideoxyhexoses and other unique sugars using gas-chromatography-mass spectrometry. *Journal of Clinical Microbiology* **28**, 546–552.
- FRENEY, J., BRUN, Y., BES, M., MEUGNIER, H., GRIMONT, F., GRIMONT, P. A. D., NERVI, C. & FLEURETTE, J. (1988). *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., two species from human clinical specimens. *International Journal of Systematic Bacteriology* **38**, 168–172.
- GARRITY, G. M., BROWN, A. & VICKERS, R. M. (1980). *Tatlockia* and *Fluoribacter*: two new genera of organisms resembling *Legionella pneumophila*. *International Journal of Systematic Bacteriology* **30**, 609–614.
- HÉBERT, G. A., MOSS, C. W., MCDUGAL, L. K., BOZEMAN, F. M., MCKINNEY, R. M. & BRENNER, D. J. (1980a). The rickettsia-like organisms TATLOCK (1943) and HEBA (1959); bacteria phenotypically similar to but genetically distinct from *Legionella pneumophila* and the WIGA bacterium. *Annals of Internal Medicine* **92**, 45–52.
- HÉBERT, G. A., STEIGERWALT, A. G. & BRENNER, D. J. (1980b). *Legionella micdadei* species nova: classification of a third species of *Legionella* associated with human pneumonia. *Current Microbiology* **3**, 255–257.
- HORI, I. (1975). Evolution of 5S rRNA. *Journal of Molecular Evolution* **7**, 75–86.
- JOHNSON, J. L. & PALLERONI, N. J. (1989). Deoxyribonucleic acid similarities among *Pseudomonas* species. *International Journal of Systematic Bacteriology* **39**, 230–235.
- JUKES, T. H. & CANTOR, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- KARR, D. E., BIBB, W. F. & MOSS, C. W. (1982). Isoprenoid quinones of the genus *Legionella*. *Journal of Clinical Microbiology* **15**, 1044–1048.
- LAMBERT, M. A. & MOSS, C. W. (1989). Cellular fatty acid compositions and isoprenoid quinone contents of 23 *Legionella* species. *Journal of Clinical Microbiology* **27**, 465–473.
- LANE, D. J., PACE, B., OLSEN, G. J., STAHL, D. A., SOGIN, M. L. & PACE, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6955–6959.
- LANE, D. J., FIELD, K. G., OLSEN, G. J. & PACE, N. R. (1988). Reverse transcriptase sequencing of ribosomal RNA for phylogenetic analysis. *Methods in Enzymology* **167**, 138–141.
- LUDWIG, W. & STACKEBRANDT, E. (1983). A phylogenetic analysis of *Legionella*. *Archives of Microbiology* **135**, 45–50.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). In *Molecular Cloning: a Laboratory Manual*, p. 438. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MORDARSKI, M., GOODFELLOW, M., WILLIAMS, S. T. & SNEATH, P. H. A. (1986). Evaluation of species groups in the genus *Streptomyces*. In *Biological, Biochemical and Biomedical Aspects of Actinomycetes*, pp. 517–526. Edited by G. Szabo, S. Biro & M. Goodfellow. Budapest: Akademiai Kiado.
- MORRIS, G. K., STEIGERWALT, A. G., FEELEY, J. C., WONG, E. S., MARTIN, W. T., PATTON, C. M., & BRENNER, D. J. (1980). *Legionella gormanii* sp. nov. *Journal of Clinical Microbiology* **12**, 718–721.
- MURRAY, R. G. E., BRENNER, D. J., COLWELL, R. R., DE VOS, P., GOODFELLOW, M., GRIMONT, P. A. D., PFENNIG, N., STACKEBRANDT, E. & ZAVARZIN, G. A. (1990). Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. *International Journal of Systematic Bacteriology* **40**, 213–215.
- ROSSAU, R., HEYNDRIKX, L. & VAN HEUVERSWEYN, H. (1988). Nucleotide sequence of a 16S ribosomal RNA gene from *Neisseria gonorrhoeae*. *Nucleic Acids Research* **16**, 6227.
- SAUNDERS, N. A. & GRANT, M. E. (1984). Elastin biosynthesis in chick embryo arteries. *Biochemical Journal* **221**, 393–400.
- SMIDA, J., KAZDA, J. & STACKEBRANDT, E. (1988). Molecular-genetic evidence for the relationship of *Mycobacterium leprae* to slow-growing pathogenic mycobacteria. *International Journal of Leprosy* **56**, 449–454.
- STACKEBRANDT, E., MURRAY, R. G. E. & TRÜPER, H. G. (1988). *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the 'purple bacteria and their relatives'. *International Journal of Systematic Bacteriology* **38**, 321–325.
- TATLOCK, H. (1944). A rickettsia-like organism recovered from guinea pigs. *Proceedings of the Society for Experimental Biology and Medicine* **57**, 95–99.
- VESEY, G., DENNIS, P. J., LEE, J. V. & WEST, A. A. (1988). Further development of simple tests to differentiate the legionellas. *Journal of Applied Bacteriology* **65**, 339–345.

- WEISBERG, W. G., DOBSON, M. E., SAMUEL, J. E., DASCH, G. A., MALLAVIA, L. P., BACA, O., MANDELCO, L., SECHREST, J. E., WEISS, E. & WOESE, C. R. (1989). Phylogenetic diversity of the Rickettsiae. *Journal of Bacteriology* **171**, 4202–4206.
- WILBUR, W. J. & LIPMAN, D. J. (1983). Rapid similarity searches of nucleic acid and protein data banks. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 726–730.
- WITT, D., LIESACK, W. & STACKEBRANDT, E. (1989). Identification of streptomycetes by 16S rRNA sequences and oligonucleotide probes. In *Recent Advances in Microbial Ecology*, pp. 679–684. Edited by T. Hattori, Y. Ishida, Y. Maruyama, R. Morita & A. Uchida. Tokyo: Japan Scientific Society Press.
- WOESE, C. R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221–271.