Identification of Species of the Genus *Legionella* Using a Cloned rRNA Gene from *Legionella pneumophila*

By NICHOLAS A. SAUNDERS,* TIMOTHY G. HARRISON,
NAFISA KACHWALLA AND ANTHONY G. TAYLOR

*Legionella Reference Unit, Division of Microbiological Reagents and Quality Control, Central
Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK*

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A cloned *Eco*RI fragment from *Legionella pneumophila*, which includes 16S and 23S rRNA genes, was used to identify bacteria belonging to the genus *Legionella* by hybridization to a series of species specific restriction fragments. Examination of the type strains of 28 species of legionellae gave different band patterns in every case. When further isolates of these species were tested the patterns obtained were usually either identical, or very similar, to those of the respective type strains. Thirty-one coded isolates were examined and of these 29 were allocated to the correct species. The remaining strains (a non-*Legionella* and a *L. pneumophila*) could not be identified using this technique. The rRNA gene probe method should be of great value in the identification of legionellae, particularly for those species which are at present very difficult to distinguish serologically.

INTRODUCTION

Legionellae are ubiquitous Gram-negative organisms, which can be isolated from most natural and man-made aquatic environments. Presumptive identification of *Legionella* species is easily made as these organisms fail to grow on conventional bacteriological culture media, but grow well on media such as buffered charcoal yeast-extract medium (BCYE), which have L-cysteine added. However, despite extensive studies of the genus, identification to the species level remains difficult.

At present there are at least 34 recognized species of *Legionella* as determined by DNA relatedness studies (Brenner, 1986). Although measurement of DNA homologies is recognized by many as the 'gold standard' in studies of bacterial taxonomy, it is both technically difficult and time-consuming. Furthermore, its use for the assignment of legionellae to the correct species requires that DNA from the unknown strain be tested against DNA of many members of the genus. Unfortunately, the biochemical and serological methods, which are of use in some cases for the identification of legionellae, do not always provide unequivocal results and consequently no effective alternative to DNA hybridization methods is currently available. Here we describe a method, based on the use of a cloned DNA fragment, to determine the species of bacterial isolates presumptively identified as belonging to the genus *Legionella*.

METHODS

Bacterial strains. Isolates of *Legionella* were cultured on BCYE agar at 37 °C for 48 h, harvested in saline (0.85% NaCl) and pelleted by centrifugation. Strains were obtained from the National Collection of Type Cultures, Colindale, UK, or are held here in the culture collection of the Legionella Reference Unit. The strains used in this study are listed in Table 1. Their identity was known from reported DNA homology data (Table 1a) or was

Abbreviation: RFLP, restriction fragment length polymorphism.

Table 1. *Legionella* strains examined in this study

(a) Strains identified by DNA homologies

Species/serogroup	NCTC*	Strain	Reference
<i>Legionella pneumophila</i>	Sgp1	11192 Philadelphia-1 ^T	Brenner <i>et al.</i> (1978)
		11286 Knoxville-1	Brenner <i>et al.</i> (1978)
		11191 Pontiac-1	Brenner <i>et al.</i> (1978)
	Sgp2	11230 Togus-1	Brenner <i>et al.</i> (1979)
	Sgp3	11232 Bloomington-2	Brenner <i>et al.</i> (1979)
	Sgp4	11233 Los-Angeles-1	KcKinney <i>et al.</i> (1979)
	Sgp5	11405 Dallas-1E	England <i>et al.</i> (1980)
	Sgp6	11406 Chicago-2	McKinney <i>et al.</i> (1980)
	Sgp7	11984 Chicago-8	Bibb <i>et al.</i> (1983)
	Sgp8	11985 Concord-3	Bissett <i>et al.</i> (1983)
	Sgp9	11986 IN-23-G1-C2	Edelstein <i>et al.</i> (1984)
	Sgp10	12000 Leiden-1	Meenhorst <i>et al.</i> (1985)
<i>Legionella anisa</i>	Sgp11	12179 797-PA-H	Thacker <i>et al.</i> (1986)
	Sgp12	12180 570-CO-H	Thacker <i>et al.</i> (1987)
<i>Legionella bozemanii</i>	Sgp1	11974 WA-316-C3 ^T	Gorman <i>et al.</i> (1985)
		11368 WIGA ^T	Brenner <i>et al.</i> (1980)
<i>Legionella cherrii</i>	Sgp2	11369 MI-15	Brenner <i>et al.</i> (1980)
		11975 Toronto-3	Tang <i>et al.</i> (1984)
<i>Legionella dumoffii</i>		11976 ORW ^T	Brenner <i>et al.</i> (1985)
		11370 NY-23 ^T	Brenner <i>et al.</i> (1980)
<i>Legionella erythra</i>		11402 Tex-KL	Brenner <i>et al.</i> (1980)
		11977 SE-32A-C8 ^T	Brenner <i>et al.</i> (1985)
<i>Legionella feeleeii</i>	Sgp1	LC217	Author's unpublished data
		12022 WO-44C ^T	Herwaldt <i>et al.</i> (1984)
<i>Legionella gormanii</i>	Sgp2	11978 691-WI-H	Thacker <i>et al.</i> (1985)
		11401 LS-13 ^T	Morris <i>et al.</i> (1980)
<i>Legionella hackeliae</i>	Sgp1	11979 Lansing-2 ^T	Brenner <i>et al.</i> (1985)
		11980 798-PA-H	Wilkinson <i>et al.</i> (1985)
<i>Legionella israelensis</i>		12010 Bercovier-4 ^T	Bercovier <i>et al.</i> (1986)
<i>Legionella jamestowniensis</i>		11981 JA-26-G1-E2 ^T	Brenner <i>et al.</i> (1985)
<i>Legionella jordanis</i>		11533 BL-540 ^T	Cherry <i>et al.</i> (1982)
<i>Legionella longbeachae</i>	Sgp1	11477 Long Beach-4 ^T	McKinney <i>et al.</i> (1981)
		11530 Tucker-1	Bibb <i>et al.</i> (1981)
<i>Legionella maceachernii</i>		11982 PX-1-G2-E2 ^T	Brenner <i>et al.</i> (1985)
<i>Legionella micdadei</i>		11371 TATLOCK ^T	Hébert <i>et al.</i> (1980)
		11372 PPA	Hébert <i>et al.</i> (1980)
		11403 HEBA	Hébert <i>et al.</i> (1980)
<i>Legionella oakridgensis</i>		11531 OR-10 ^T	Orrison <i>et al.</i> (1983)
<i>Legionella parisiensis</i>		11983 PF-209C-C2 ^T	Brenner <i>et al.</i> (1985)
<i>Legionella rubrilucens</i>		11987 WA-270A-C2 ^T	Brenner <i>et al.</i> (1985)
		LC215	Author's unpublished data
		LC216	Author's unpublished data
<i>Legionella saintelensis</i>		11988 Mt St Helens-4 ^T	Campbell <i>et al.</i> (1984)
<i>Legionella santicrucis</i>		11989 SC-63-C7 ^T	Brenner <i>et al.</i> (1985)
<i>Legionella spiritensis</i>		11990 Mt St Helens-9 ^T	Brenner <i>et al.</i> (1985)
		12082 ML 76	Author's unpublished data
<i>Legionella steigerwaltii</i>		11991 SC-18-C9 ^T	Brenner <i>et al.</i> (1985)
<i>Legionella wadsworthii</i>		11532 81-716A ^T	Edelstein <i>et al.</i> (1982)
Unnamed <i>Legionella</i> species A		51-83 (1308)	P. J. L. Dennis, pers. comm.
Unnamed <i>Legionella</i> species B		59-84 (1477)	P. J. L. Dennis, pers. comm.
Unnamed <i>Legionella</i> species C		199-82 (1224)	P. J. L. Dennis, pers. comm.
Unnamed <i>Legionella</i> species D		200-83 (1335)	P. J. L. Dennis, pers. comm.
Unnamed <i>Legionella</i> species E		95-83 (1347)	P. J. L. Dennis, pers. comm.

Table 1 (continued)

(b) Strains identified by biochemical and serological methods†

Species/serogroup	NCTC*	Strain	Source‡
<i>Legionella pneumophila</i>	Sgp1	11404 Bellingham-1	
		LC20	Clinical isolate/France
	Sgp5	11417 LC28	Clinical isolate/France
		LC434	Environmental isolate/UK
	Sgp6	11287 Oxford-1	
		LC204	Environmental isolate/UK
	Sgp12	LC205	Environmental isolate/UK
		82A-3105	
<i>Legionella anisa</i>	Sgp13	LC419	Environmental isolate/Singapore
		LC213	Environmental isolate/France
	Sgp?§	LC318	Environmental isolate/UK
		LC319	Environmental isolate/UK
		LC320	Environmental isolate/UK
		LC329	Environmental isolate/UK
		LC377	Environmental isolate/UK
		LRU52	Environmental isolate/UK
		LRU68	Environmental isolate/UK
		LRU56	Environmental isolate/UK
<i>Legionella bozemanii</i>	Sgp1	LRU81	Environmental isolate/UK
		LRU82	Clinical isolate/UK
		LRU83	Clinical isolate/UK
		LRU85	Clinical isolate/UK
<i>Legionella dumoffii</i>		LC455	Environmental isolate/Singapore
<i>Legionella gormanii</i>		LC458	Environmental isolate/Singapore
<i>Legionella wadsworthii</i>		LC121	Environmental isolate/France
Unnamed <i>Legionella</i> species C		LC218a	Environmental isolate/UK
		LC221a	Environmental isolate/UK
<i>Pseudomonas vesicularis</i>		ML81	Environmental isolate

†, Type strain of the species.

* Catalogue numbers of strains available from the National Collection of Type Cultures (NCTC), 61 Colindale Avenue, London NW9 5HT, UK. Where strains are not available from the NCTC they are held in the culture collection of the Legionella Reference Unit.

† Brenner *et al.* (1985).

‡ Strains bracketed together are epidemiologically related.

§ Serological variant of undetermined serogroup.

established using biochemical and serological techniques (Table 1b) (Brenner *et al.*, 1985). An environmental isolate submitted to this laboratory, as a possible *Legionella*, was shown by the use of a API 20ne identification kit to be a strain of *Pseudomonas vesicularis*. This strain was included in the panel of coded isolates.

For this study strains were considered to be epidemiologically related to each other if they were obtained from the same environmental source (although not necessarily at the same time), or were obtained from a patient exposed to that source. Strains for which there was no obvious link were considered to be epidemiologically unrelated.

Construction of a *L. pneumophila* genome library. DNA isolated from the Knoxville-1 strain of *L. pneumophila* was used to prepare a gene library in the vector λ gtWES. λ B. (Tiemeier *et al.*, 1976) using methods described by Maniatis *et al.* (1982). The λ B. fragment was excised with *Eco*RI and replaced by *Eco*RI restriction fragments (2–15 kb) derived by complete digestion of the genomic DNA. Recombinant phage, resulting from *in vitro* packaging, were plated on *E. coli* NM531 (*supE supF hsdR recA*) (Kaiser & Murray, 1985).

Preparation of 16S and 23S rRNA subunits. *L. pneumophila* (Knoxville-1) cells were treated with lysozyme (3.3 mg ml⁻¹) for 30 min at 37 °C in buffer containing 25% (w/v) sucrose, 50 mM-Tris/HCl, pH 8.0. The cells were recovered by centrifugation and RNA was prepared (Saunders & Grant, 1984). The 16S and 23S rRNA subunits were purified on a linear 5–20% (w/v) sucrose gradient in 10 mM-HEPES/NaOH, pH 7.5, by centrifugation at 125000 g_{av} for 16 h in a SW60Ti rotor (Beckman).

Screening for rRNA genes. The library was screened by plaque filter hybridization (Maniatis *et al.*, 1982) on nylon filters (Hybond-N; Amersham). The probe was prepared from a mixture of 16S and 23S rRNAs derived from *L. pneumophila* (Knoxville-1) which were subjected to polynucleotide kinase labelling with [γ -³²P]ATP (Amersham) following partial alkaline hydrolysis (Maniatis *et al.*, 1982).

Preparation of bacterial DNA. DNA was extracted using a propan-2-ol precipitation method (Owen & Borman, 1987). Briefly, the pellet of bacterial cells was resuspended in buffer containing 150 mM-NaCl, 15 mM-EDTA, 50 mM-Tris/HCl, pH 8.0 and RNAase A ($50 \mu\text{g ml}^{-1}$; Sigma) and lysed by addition of SDS to 1% (w/v). The lysate was allowed to clear at 37°C for 15–30 min and then proteinase K (Sigma) was added (0.1 mg ml^{-1}) and incubated for a further 30 min. The protease treated lysate was then extracted with chloroform:isoamyl alcohol (24:1, v/v) and made to 2.5 M in ammonium acetate by addition of a 7.5 M stock solution. The extracted DNA was precipitated by addition of propan-2-ol (0.54 vols), collected by centrifugation and washed twice in 70% (v/v) ethanol. The final pellet was dried *in vacuo* and resuspended in TE buffer (10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA).

Restriction endonuclease digestion and agarose-gel electrophoresis. Bacterial DNA ($1\text{--}2 \mu\text{g}$) was digested with *NciI* (2–4 units) (Northumbria Biologicals), or *HaeIII* (2–4 units) (Amersham) in medium salt buffer (Maniatis *et al.*, 1982) for 2 h at 37°C . The restriction fragments were separated by electrophoresis in 0.8% (w/v) or 1.2% (w/v) agarose gels at 20 V (1 V cm^{-1}) for 18 h in Tris/borate buffer (0.089 M-Tris, 0.089 M-boric acid, 0.002 M-EDTA) containing $0.5 \mu\text{g}$ ethidium bromide ml^{-1} . Bacteriophage λ (strain c1857 ts *Sam7*) DNA fragments, derived by cleavage with *PstI* and *EcoRI*, were run as molecular size standards. Following electrophoresis the gel was soaked for 1 h each in denaturing solution (0.5 M-NaOH, 1.5 M-NaCl) and then in neutralizing solution (1 M-Tris/HCl, pH 8.0, 1.5 M-NaCl).

Southern transfer of DNA samples. Southern transfer of DNA was done as described by Maniatis *et al.* (1982) using nylon filters (Hybond-N; Amersham). The filters were air-dried, covered in Mobilrapper II (Mobil Plastics, Virton, Belgium) and exposed to longwave UV light (3 min) from a transilluminator (UV Products) to effect covalent binding of the DNA fragments to the solid phase.

Preparation of λ phage DNA. Bacteriophage particles were purified from a lysed culture by the pelleting method (Maniatis *et al.*, 1982). DNA was released from phage, suspended in SM buffer (50 mM-Tris/HCl, pH 7.5, 0.1 M-NaCl, 10 mM-MgSO₄ and 0.01% w/v, gelatin), by addition of 0.5 M-EDTA (pH 8.0) to 20 mM, proteinase K to $50 \mu\text{g ml}^{-1}$, SDS (20% w/v) to 0.5% followed by incubation at 65°C for 1 h. The DNA was purified by successive extractions with phenol, phenol/chloroform/isoamyl alcohol (25:24:1, by vol. and chloroform/isoamyl alcohol (24:9, v/v). The DNA in the aqueous phase was precipitated with ethanol in the presence of 0.3 M-sodium acetate, pH 6.0, washed twice with 70% (v/v) ethanol and dried *in vacuo*. At this stage the λ DNA was unsuitable for restriction endonuclease digestion or for labelling reactions. Contaminating materials were removed by further treatment with RNAase and proteinase K followed by extraction with chloroform and precipitation with propan-2-ol as described above for the purification of bacterial DNAs. In our hands this modified procedure gives high yields of λ DNA [$>1 \mu\text{g (ml culture)}^{-1}$] which is both pure, as determined by gel electrophoresis followed by ethidium bromide staining, and a good substrate for restriction endonucleases and DNA polymerase I.

Labelling and hybridization of probe. λ 142 DNA was labelled by primer extension using biotin-11-dUTP (Gibco). The labelled DNA was separated from unincorporated dNTPs by spun column chromatography (Maniatis *et al.*, 1982). Southern blots were prehybridized for 3 h and then hybridized for 18 h at 42°C in hybridization solution [45% v/v, formamide, $5 \times$ SSPE (1 $\times$ SSPE is 0.15 M-NaCl, 1 mM-EDTA, 10 mM-sodium phosphate/NaOH, pH 7.4), $5 \times$ Denhardt's solution (1 $\times$ Denhardt's solution is 0.02% BSA, 0.02% PVP, 0.02% Ficoll), 0.1% w/v, SDS, 100 μg denatured herring sperm DNA ml^{-1}] containing labelled probes (150 ng), denatured by heating (100°C for 5 min). Following incubation the filters were washed at low-stringency in $2 \times$ SSC, 0.1% (w/v) SDS at 50°C ($2 \times 15 \text{ min}$ and $1 \times 30 \text{ min}$) as described in Maniatis *et al.* (1982). The filters were then air-dried and hybridized sequences detected using the Blu-gene system (Gibco).

Northern Blotting. RNA from *L. pneumophila* (Knoxville-1) was subjected to agarose-gel electrophoresis (1% w/v, gel) in the presence of formaldehyde and blotted onto nylon filters (Maniatis *et al.*, 1982). The blot was hybridized to λ 142, which had been labelled by nick-translation (Rigby *et al.*, 1977) with [α - ^{32}P]dCTP (Amersham). The bound probe was detected by autoradiography using Kodak X-Omat AR film and intensifying screens.

DNA fragment size estimation. The molecular size (in base pairs) of restriction fragments was calculated from a standard curve of relative mobility against molecular size of the *EcoRI* and *PstI* restriction fragments of λ phage, which were run on each gel.

RESULTS

Selection and characterization of a rRNA gene probe

When the *L. pneumophila* gene library was screened with end-labelled rRNA fragments, approximately 0.5% of the clones gave a strong positive signal indicating hybridization to cloned rRNA sequences. One of these clones (λ 142) was purified and used to probe a Northern blot of *L. pneumophila* RNA. Both the 23S and 16S rRNA species hybridized to the probe and gave a signal proportional to the quantity of rRNA subunits in the RNA preparation (Fig. 1). The smaller RNA molecules including 5S and tRNA were removed by washing with 3 M-sodium

Fig. 1. Northern blot analysis of λ 142. Total RNA from *L. pneumophila* Knoxville-1 (5 μ g) was run on a 1% agarose gel, then blotted and hybridized to 32 P-labelled λ 142; following washing under stringent conditions the bands were visualized by autoradiography.

acetate during preparation; consequently, it is not known whether sequences encoding these subunits are present on λ 142. These results demonstrate the proximity of the two genes and suggest that the rRNA genes of *L. pneumophila* are clustered. If this is the case then λ 142 must include the complete sequence of at least one ribosomal subunit since the cloned fragment is larger than a single rRNA gene cluster. It is also likely that a high proportion of the complete sequence of both major rRNA molecules is present since the hybridization signal obtained was proportional to the quantity of 16S and 23S subunits in the RNA preparation.

Restriction endonuclease digestion of DNA from legionellae

DNA preparations from all isolates in Table 1, except the three strains of *L. micdadei*, were efficiently cleaved by the restriction endonuclease *Nci*I. Although resistant to digestion with *Nci*I the *L. micdadei* DNAs were susceptible to digestion with *Hae*III (see Fig. 3). The DNA preparations obtained from the isolate of *Legionella* species A appeared to be highly susceptible to *Nci*I yielding a very high proportion of small restriction fragments. This suggests that the mol% G + C of *Legionella* species A is relatively high. A similar result was observed for the DNA from *P. vesicularis*, which has a mol% G + C of 65.8 (Palleroni, 1984).

Analysis of the restriction fragment patterns obtained

Examination of the restriction fragments detected by probing the blots of *Nci*I or *Hae*III digested DNAs revealed these to be species specific. Thus distinct patterns were obtained for the 28 type strains examined. A selection of these patterns is shown in Fig. 2 (*Nci*I) and Fig. 3 (*Hae*III). None of the 81 isolates examined gave the same pattern as another isolate belonging to a different species. Epidemiologically distinct isolates belonging to the same species gave patterns which were indistinguishable from those of the type strain in the cases of *L. pneumophila* (five isolates), *L. anisa* (five isolates), *L. micdadei* (two isolates), *L. feeleii* (one isolate), *L.*

Fig. 2. *Nci*I restriction fragments from *Legionella* species detected with the probe λ 142. Restriction fragments (1 μ g per track), derived by *Nci*I digestion of DNA from *Legionella* species, were subjected to electrophoresis on a 0.8% agarose gel at 1 V cm^{-1} for 16 h. The DNA was blotted and hybridized to biotinylated λ 142 (150 ng). Bound probe was visualized using the Blu-gene kit reagents. Tracks 1–20 show the patterns obtained for 19 strains as follows: 1, *L. pneumophila* Knoxville-1; 2, *L. pneumophila* LC20; 3, *L. longbeachae* Long Beach-4; 4, *L. longbeachae* Tucker-1; 5, *L. sainthelensi* Mt St Helens-4; 6, *L. spiritensis* Mt St Helens-9; 7, *L. oakridgensis* OR-10; 8, *L. bozemanii* WIGA; 9, *L. bozemanii* MI-15; 10, *L. bozemanii* Toronto-3; 11, *L. anisa* WA-316-C3; 12, *L. gormanii* LS-13; 13, *L. dumoffii* NY-23; 14, *L. dumoffii* Tex-KL; 15, *L. steigerwaltii* SC-18-C9; 16, *L. cherrii* ORW; 17, *L. israelensis* Bercovier-4; 18 and 19, *L. hackeliae* Lansing-2; 20, *L. hackeliae* 798-PA-H. Restriction fragments of λ phage (2 ng) are shown in track 21.

hackeliae (one isolate), *L. spiritensis* (one isolate), *Legionella* species C (one isolate), *L. wadsworthii* (one isolate), *L. dumoffii* (one isolate), *L. gormanii* (one isolate), or differed in the position of a single band, or in the presence/absence of a single band, in the cases of *L. rubrilucens* (two isolates), *L. dumoffii* (one isolate), *L. longbeachae* (one isolate), *L. erythra* (one isolate).

In the case of *L. bozemanii* strains MI-15 and Toronto-3 (serogroup 2) both showed two restriction fragment length polymorphisms (RFLPs) from the type strain WIGA, and a further group of related isolates (LRU81, LRU82, LRU83 and LRU85) shared the same pattern as WIGA. This diversity in pattern might be expected from the low sequence homology in this species (Brenner *et al.*, 1980).

Examination of 22 strains of *L. pneumophila* belonging to the 13 recognized serogroups provided the best opportunity to assess the degree of variation between restriction sites within a species. Fig. 4 shows the results of analysis of 15 strains (the banding patterns of the other seven strains are not presented here). All 22 strains had the same pattern of bands of molecular size <0.8 kbp and also shared a fragment at 2.8 kbp. Twenty of the strains also had bands at 2.0 kbp and 19 of these had a further restriction fragment at 3.0 kbp. A 1.8 kbp band was present in 15 strains. Six strains, including the type strain of *L. pneumophila* (Philadelphia-1), had only these four bands (1.8, 2.0, 2.8 and 3.0 kbp) of >0.8 kbp. Track 9 (Fig. 4) shows an example of this pattern. Four more strains had the pattern described above but with one additional band at 2.3 kbp. This group included Knoxville-1, the strain from which the probe sequence was derived (track 2). A further four isolates had all of the type strain bands together with other variable features. Five of the remaining strains had the 2.0, 2.8 and 3.0 kbp fragments and at least one other band. The serogroup 11 type strain (797-PA-H), shown in track 12, gave a pattern including the 1.8, 2.0 and 2.8 kbp species together with two other major bands. Two strains gave

Fig 3. *Hae*III restriction fragments from *Legionella* species detected with the probe λ 142. Restriction fragments (1 μ g per track), derived by *Hae*III digestion of DNA from *Legionella* species, were subjected to electrophoresis on a 1.2% agarose gel at 1 V cm^{-1} for 16 h. The DNA was blotted and hybridized to biotinylated λ 142 (150 ng). Bound probe was visualized using the Blu-gene kit reagents. Tracks 2-13 show the patterns obtained for 12 strains as follows: 2, *L. micdadei* PPA; 3, *L. micdadei* HEBA; 4, *L. micdadei* TATLOCK; 5, *L. maceachernii* PX-1-G2-E2; 6, *L. cherrii* ORW; 7, *L. pneumophila* Knoxville-1; 8, *L. bozemanii* MI-15; 9, *L. bozemanii* WIGA; 10, *L. bozemanii* Toronto-3; 11, *L. anisa* WA-316-C3; 12, *L. parisiensis* PF-209C-C2; 13, *L. steigerwaltii* SC-18-C9. Restriction fragments of λ phage are shown in tracks 1 (2 ng) and 14 (1 ng).

rather different patterns having only the <0.8 and 2.8 kbp bands in common with Philadelphia-1. These isolates (Los Angeles-1 and Dallas-1E) shared two other fragments at 1.9 and 2.3 kbp.

Identification of 'unknown' legionellae

To determine whether the method could be used to identify the species of a putative legionella, 31 isolates (which had been examined previously) were coded by one of us (T.G.H.) and identification was then attempted (by N.A.S.). The pattern obtained for each coded isolate was compared against those previously obtained for each species and thus the identity of the coded isolate was determined. All of the coded isolates were susceptible to digestion with *Nci*I. Of the 31 strains 23 were epidemiologically unrelated. Seventeen of these isolates were indistinguishable on the basis of restriction fragments from previously examined strains of known identity. These isolates were correctly identified as *L. pneumophila* (four isolates), *L. anisa* (six isolates), *L. bozemanii* (one isolate), *Legionella* species C (one isolate), *L. spiritensis* (one isolate) *L. wadsworthii* (one isolate), *L. dumoffii* (one isolate) and *L. gormanii* (one isolate). Five other isolates gave patterns similar to but distinct from those of type strains of various *Legionella* species. Two of these were correctly identified as *L. pneumophila* on the basis of the presence of bands at 2.0, 2.8 and 3.0 kbp. Another pair of isolates were identified as *L. rubrilucens*, the restriction fragments differing only in the position of a single band, compared with those from *L. rubrilucens* (WA-270A-C2). The fifth isolate, previously identified serologically as *L. rubrilucens*,

Fig. 4. Analysis of 15 strains of *L. pneumophila*. Restriction fragments (1 µg per track), derived by *Nci*I digestion of DNA from *L. pneumophila*, were subjected to electrophoresis on a 0.8% agarose gel at 1 V cm⁻¹ for 16 h. The DNA was blotted and hybridized to biotinylated λ142 (150 ng). Bound probe was visualized using the Blu-gene kit reagents. Tracks 2–16 show the patterns obtained for 15 strains as follows: 2, Knoxville-1; 3, Togus-1; 4, Bloomington-2; 5, Los Angeles-1; 6, Cambridge-2; 7, Oxford-1; 8, Chicago-8; 9, Concord-3; 10, IN-23-G1-C2; 11, Leiden-1; 12, 797-PA-H; 13, Dallas-1E; 14, LC28; 15, LC20; 16, Bellingham-1. Restriction fragments of λ phage (2 ng) are shown in tracks 1 and 17.

gave a pattern of fragments at <0.8 kbp, which was consistent with either of the recognized red autofluorescent species (*L. rubrilucens* or *L. erythra*). However, this strain (LC217) was identified as *L. erythra* on the basis of the positioning of bands of higher molecular size. This identification was subsequently confirmed by DNA/DNA hybridization studies, using a filter method (Johnson, 1981). LC217 showed 96% homology with *L. erythra* (SE-32A-C8) DNA at low stringency (in the same experiment three *L. rubrilucens* strains – WA-270A-C2, LC215 and LC216 – gave values of 35 to 42% homology to *L. erythra*). At 10 °C below the melting temperature of *L. erythra* DNA (stringent conditions) the homology value dropped to 83% (*L. rubrilucens* 9 to 13%). Two isolates could not be assigned to a species.

Five groups of epidemiologically related strains were examined. These comprised *L. pneumophila* (two isolates), *L. anisa* (two groups of two and three isolates each), *L. bozemanii* (four isolates), *Legionella* species C (two isolates). The isolates within each group gave indistinguishable patterns.

DISCUSSION

Ribosomal RNA genes are highly conserved and present in multiple copies in the chromosomes of bacterial species. These properties make them particularly suitable for the studies presented here. The conserved nature of the rRNAs allows a single probe to be used in studies of the entire genus (*Legionella*) and offers the possibility that species-specific patterns will be obtained. The presence of multiple copies of the rRNA genes increases the sensitivity, and power to discriminate between species, of the method. A similar procedure based on the use of 5'-end-³²P-labelled *E. coli* 16S and 23S rRNA as a probe has been published (Grimont *et al.*, 1987). The cloned rRNA gene used in the study presented here has several advantages over the use of rRNA subunits as a probe. Firstly, DNA cloned in λ can readily be purified in quantity. The method used here compares favourably with the lengthy procedure for the preparation of

rRNA subunits. Secondly, the cloned DNA is of highly consistent quality. Thirdly, since the cloned fragment appears to represent a cluster of rRNA genes there is likely to be an equal proportion of sequences for the rRNA subunits (5S, 16S and 23S) available for hybridization to target sequences. Fourthly, the vector sequences of the probe hybridize to λ fragments run on tracks adjacent to samples of *Legionella* DNA and these provide ideal molecular size standards allowing the species patterns to be compared from one gel to another. A possible disadvantage of the cloned probe used in this study is the presence of DNA sequences flanking the rRNA genes. However, it was considered that these sequences would not hybridize to species other than *L. pneumophila* because of the low DNA homologies between *Legionella* species. In the case of *L. pneumophila* the flanking sequences could cause some variation in the patterns due to their hybridization to non-rRNA gene sequences. In practice this did not appear to be a serious problem (Fig. 4).

Although DNA preparations from 27 *Legionella* species were satisfactorily digested with *Nci*I, DNA from all three *L. micdadei* strains was refractory to cleavage with this enzyme. This is probably because of methylation of the DNA in this species at the internal cytosine residue of the *Nci*I site. One of these strains (TATLOCK) has been shown to have a restriction enzyme activity (Chen *et al.*, 1986), which presumably acts at the *Nci*I recognition site.

*Nci*I recognizes the sequence 5'-CC₃GG-3' and was expected to have many sites of cleavage within the rRNA gene cluster of λ 142. This consideration was critical since intra-species sequence variations within the highly conserved rRNA genes are relatively uncommon whilst the sequences of the flanking DNA would be expected to be more variable. In this regard it is expected that the method using *Nci*I described here will be more useful than that described by P. A. D. Grimont and colleagues for *Legionella* species identification, where restriction endonucleases having hexanucleotide recognition sequences are used (Grimont & Grimont, 1986; Grimont *et al.*, 1987). A method based on sequence differences in the flanking DNA (as would be the case when the recognition sites of the restriction enzyme chosen are widely spaced) would be expected to yield a greater proportion of intra-species differences in patterns of restriction fragments. This would be undesirable since it would then be difficult to identify unknown isolates. It might be possible to further reduce the influence of the variable flanking sequences by using a nuclease which recognizes many sites within the rRNA gene cluster. This is demonstrated in Fig. 3 for *Hae*III; however, the small restriction fragments thus generated yield relatively unclear patterns which are difficult to interpret.

The results obtained using the restriction fragment length analysis technique with the rRNA gene probe on a number of isolates of *L. pneumophila* shows that some restriction sites are conserved whilst others are more variable in position, suggesting that some of the sequences hybridizing to the probe are more variable than others. It would be expected that these non-conserved restriction sites correspond either to the flanking sequences or to parts of the rRNA gene which are less conserved. Variable sequences are known to be present in parts of the bacterial 16S rRNA sequence (Woese *et al.*, 1983). It is worthy of note that studies of DNA relatedness and isoenzyme patterns indicate that *L. pneumophila* is a relatively diverse species at both the genotypic and phenotypic levels (Brenner *et al.*, 1978; Selander *et al.*, 1985). Some evidence was obtained in this study that the more distantly related strains of *L. pneumophila* give rise to divergent patterns. For example, Los Angeles-1 and Dallas-1E (Fig. 4, tracks 5 and 13), two strains belonging to a subset of isolates described as Species 1 by Selander *et al.* (1985), gave similar patterns of restriction fragments which were distinct from those of the other isolates of *L. pneumophila* examined.

Comparison of the results obtained for the 22 *L. pneumophila* strains examined in this study, with those of previous work involving 48 serogroup 1 strains of this species, probed with a non-ribosomal sequence (Saunders *et al.*, 1987), shows the advantage of using rRNA sequences as probes for species identification. Many fewer RFLPs are revealed by the rRNA gene probe sequence and consequently less intra-species pattern variations occur.

The results of testing 'unknown' coded isolates show the utility of this method. In most cases (24 out of 31) the pattern obtained was indistinguishable from that of at least one strain, of

known identity, which had been examined previously. In these cases the unknown was accurately identified. In three further instances the pattern differed in the position of a single band and it was possible to assign these isolates to the correct species with a high level of confidence. In two instances the patterns obtained from the DNA of 'unknowns' had strong similarities to the pattern obtained from an isolate of a known species and the tentative identification of these strains was found to be correct. In practice, positive identification of isolates in this category will rely on further data. Two isolates were not assigned to a species. One, a non-*Legionella* species, was subsequently shown to be an isolate of *Pseudomonas vesicularis*. The second, a serologically unusual environmental isolate from Singapore, was characterized as a *L. pneumophila* strain on the basis of biochemical tests and its reaction with a species specific monoclonal antibody (Genetic Systems). The restriction fragment pattern obtained was unusual, although the bands typical of this species at 3.0, 2.0 and <0.8 kbp were present, fragments of 2.8 and 1.8 kbp in size were absent. No DNA/DNA hybridization data are available for this isolate, but it may be that both the atypical restriction fragment pattern and serological results obtained correlate to low genome homology with the type strain. However, it must be recognized that a novel pattern of bands could result from analysis of a *Legionella* isolate with subtle base sequence differences compared with previously examined isolates of that species. This is because RFLPs can result from a single base deletion or substitution. Alternatively, a novel pattern can represent a previously unreported *Legionella* species or a non-*Legionella* isolate. Consequently a novel pattern yields little useful information.

It is clear that the method described in this paper will be of considerable value in studies on the legionellae and should provide a valuable complement to both the current biochemical and serological methods (which may provide inconclusive data), and DNA/DNA hybridization, which is technically demanding and time-consuming. For example, using the probe, it was relatively simple to distinguish between the blue-white autofluorescent species, *L. anisa*, *L. bozemanii*, *L. steigerwaltii*, *L. cherrii*, *L. dumoffii*, *L. gormanii* and *L. parisiensis*. Among these species it is particularly useful to be able to distinguish *L. anisa* from *L. bozemanii* and *L. steigerwaltii* from *L. cherrii* as these pairs of genospecies can otherwise be reliably split only by measurement of percentage divergence in DNA hybridization studies. It is anticipated that as more strains are examined and the database of RFLPs expands, a greater proportion of isolates will be identified with a high degree of confidence.

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