International Journal of Systematic Bacteriology (1998), **48**, 851–858 Printed in Great Britain

Leptospira fainei sp. nov., isolated from pigs in Australia

P. Perolat,[1] R. J. Chappel,[2] B. Adler,[3] G. Baranton,[4] D. M. Bulach,[3] M. L. Billinghurst,[2] M. Letocart,[1] F. Merien[1] and M. S. Serrano[3]

Author for correspondence: P. Perolat. Tel: +687 27 02 80. Fax: +687 27 33 90. e-mail: perolat_pasteur@canl.nc

[1] *Leptospira* Laboratory, Institut Pasteur, Nouméa, New Caledonia

[2] Victorian Institute of Animal Science, Attwood, Victoria, Australia

[3] Department of Microbiology, Monash University, Clayton, Victoria, Australia

[4] WHO Collaborating Centre on Epidemiology of Leptospirosis, Molecular and Medical Bacteriology Unit, Institut Pasteur, Paris, France

Pathogenic leptospires can be causative agents of reproductive problems in pigs. Cultures of uteri and kidneys from two pig herds in New South Wales and Victoria (Australia) yielded five strains identified as *Leptospira* on morphological and cultural grounds. Phenotypic characteristics (growth at 13 and 30 °C, growth in the presence of 8-azaguanine) were intermediate between those of pathogenic and saprophytic leptospires. No cross-agglutination was observed with reference antisera representing the 24 pathogenic serogroups and the main saprophytic ones. Antiserum against one of the strains did not agglutinate reference strains representative of any serogroup. This provided evidence of a new serovar, designated hurstbridge. Genomic characterization of the five strains was achieved using five molecular approaches. Mapped restriction site polymorphisms in the *rrs* (16S rRNA) gene were not related to those of any reference strains. Arbitrarily primed PCR fingerprints suggested clonality of the five strains. The strains all showed an identical and unique PFGE profile. PCR, using primers specific for the *rrs* gene of pathogenic leptospires, amplified corresponding sequences from the strains. DNA–DNA hybridization (and reciprocal experiments) using the S1 nuclease/TCA method was performed between one of the strains and the reference strains of *Leptospira* species. The homology ranged from 0 to 36% (the latter being with *Leptospira inadai*) thus satisfying the criterion of a new species, *Leptospira fainei* (type strain BUT 6^T). Phylogenetic analysis of 16S rRNA sequences showed that *L. fainei* and *L. inadai* formed a clade separate from the previously recognized 'saprophyte' and 'pathogen' clades.

Keywords: *Leptospira fainei* sp. nov., pigs, phylogenetic analysis, serovar hurstbridge, molecular typing

INTRODUCTION

Leptospirosis, caused by pathogenic serovars of *Leptospira*, is a zoonosis of worldwide distribution. For taxonomic purposes, and as an aid to epidemiological studies, the pathogenic leptospires have been subdivided into serovars by agglutination–absorption patterns (4). Related serovars have been placed within 24 recognized serogroups, containing more than 200 serovars (7). Alternative serological typing systems based on either monoclonal antibodies or factor analysis give comparable results to conventional serotyping methods (4, 10).

Molecular taxonomic studies have greatly improved our knowledge of the diversity of *Leptospira*. DNA–DNA hybridization studies (19, 24) showed that members of *Leptospira interrogans sensu lato* were diverse at the DNA level. Subsequently, leptospires were grouped into seven (24) and then eight (19) pathogenic species (*Leptospira borgpetersenii*, *Leptospira inadai*, *Leptospira interrogans sensu stricto*, *Leptospira kirschneri*, *Leptospira meyeri*, *Leptospira noguchii*, *Leptospira santarosai* and *Leptospira weilii*) and two saprophytic ones (*Leptospira biflexa* and *Leptospira wolbachii*).

Abbreviations: AP-PCR, arbitrarily primed PCR; MRSP, mapped restriction site polymorphism; SCT, Subcommittee on Taxonomy of *Leptospira*.

The GenBank accession number for the 16S rRNA sequence of *L. fainei* is U60594.

Several molecular techniques can be used as additional typing systems in epidemiological studies, since they identify strain differences at the infraspecific level. These include restriction endonuclease analysis of chromosomal DNA by fixed-field electrophoresis (6) or PFGE (9), RFLPs (25) and ribotyping (16). Nevertheless, the identification of leptospires at the species level remains difficult, as DNA–DNA hybridization is not feasible for routine identification. PCR-based strategies were therefore developed to categorize the new species of *Leptospira* (18). Mapped restriction site polymorphisms (MRSPs) in PCR-amplified ribosomal genes grouped reference strains in agreement with their DNA relatedness (17, 18). Arbitrarily primed PCR (AP-PCR) generates distinctive fingerprints that can be used for molecular epidemiology (17) and to rapidly identify strains at the species level (14).

In 1994, the Victorian Institute of Animal Science (Attwood, Victoria, Australia) isolated five *Leptospira* strains that did not belong to any known serogroup (3). These strains were isolated from cultures of uteri and kidneys from two pig herds, in Victoria and New South Wales, and were identified as a new serovar named hurstbridge. Examination by conventional phenotyping and molecular typing methods suggested that these strains were identical and represented a new species within the genus *Leptospira*. This was confirmed by DNA–DNA hybridization experiments, and the name of *Leptospira fainei* sp. nov. is proposed for this organism. In this paper, we report the antigenic and genetic characterization and phylogenetic position of *L. fainei*.

METHODS

Bacterial strains and reference antisera. All reference strains used were from the WHO Reference Laboratory and Collaborating Centre for Leptospirosis, Institut Pasteur, Paris.

For conventional serotyping, the reference antisera of the 24 pathogenic serogroups (Australis, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Lyme, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum, Sarmin, Sejroe, Shermani and Tarassovi) and of the two main saprophytic serogroups (Codice and Semaranga) were tested according to the recommendations of the Subcommittee on Taxonomy of *Leptospira* (SCT) (13). A reference antiserum specific for hurstbridge reference strain BUT 6^T was produced in New Zealand White rabbits according to SCT recommendations (7, 10) and used for reverse agglutination tests (7) against each reference strain representative of the serogroups listed above.

DNA–DNA hybridization experiments involved (i) reference strains representing the eight pathogenic species, (ii) reference strains representing the two saprophytic species and (iii) the type strain of the closely related genus *Turneria*.

The five hurstbridge strains (BUT 6^T, BUT 8, BKID 6, BKID 7 and WKID) (3) were included in all the phenotypic and genotypic tests, except in DNA–DNA hybridization, where BUT 6^T, the designated reference strain of serovar hurstbridge, and WKID were tested. For 16S rRNA gene sequencing, only BUT 6^T was studied as the reference strain.

Culture conditions and preparation of DNA. Strains were grown to stationary phase in EMJH medium (5) at 30 °C with shaking. Chromosomal DNA was extracted using the phenol/chloroform method (2).

Phenotypic characterization

(i) Light and electron microscopy. Isolates were examined by dark-field microscopy. One strain (BUT 6^T) was examined by transmission electron microscopy using a Philips CM12 STEM electron microscope, employing negative staining with 2% phosphotungstic acid, at a final magnification of $\times 9450$.

(ii) Growth characteristics. Isolates were tested for characteristics that differentiate pathogenic and saprophytic leptospires (10, 11), with *L. interrogans* serovar icterohaemorrhagiae (strain RGA^T) and *L. biflexa* serovar patoc (strain Patoc I^T), respectively, as control pathogenic and saprophytic strains. In addition, *L. inadai* serovar lyme (strain 10^T) was included as a leptospiral strain with intermediate growth characteristics (24).

The ability to grow at 13 and 30 °C was tested in duplicate. Growth was assessed by dark-field microscopy on days 0, 3, 7, 10 and 13. Viability of strains which did not grow at 13 °C was then confirmed by subculture at 30 °C.

Growth in the presence of 8-azaguanine (225 μg ml^{-1}) was tested (12) in duplicate at 30 °C and assessed as above. Viability of strains which did not grow in 8-azaguanine was confirmed by simultaneous culture at 30 °C in EMJH medium without 8-azaguanine.

(iii) Serotyping. Serotyping of hurstbridge strains was performed according to the recommendations of the SCT (7, 10), using antisera against strain BUT 6^T and against reference strains representative of the 26 serogroups listed above.

Genetic characterization

(i) AP-PCR. AP-PCR was performed with the purified total genomic DNA as described previously (17, 18) using the primers KF (5′-CAC GCA CAC GCA CAG AGA-3′), KG (5′-CAC ACG CAC ACG GAA GAA-3′), KN (5′-CCT TGC GCG CAT GTA CAT GG-3′), KpnR (5′-CCA AGT CGA CAT GGC ACR TGT ATA CAT AYG TAA C-3′), KZ (5′-CCC ATG TGT ACG CGT GTG GG-3′), RSP (5′-GGA AAC AGC TAT GAC CAT GA-3′), SP (5′-TTG TAA AAC GAC GGC CAG-3′), PR1 (5′-CGC TAA AAC TAA TAT CAT GA-3′) and PR2 (5′-GGA AAC TTA CAC TAA CAG-3′). Briefly, AP-PCR reactions were cycled twice (GeneAmp 9600; Perkin Elmer) with a low-stringency profile (5 min at 94 °C, 5 min at 40 °C and 5 min at 72 °C) followed by 40 cycles at high stringency (1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C). PCR products were analysed using urea sequencing gels autoradiographed for 24–48 h on Kodak X-Omat X-ray film.

(ii) PFGE. Restriction digestion with *Not*I (Amersham) and PFGE of *in situ* lysed cells was performed using a Pulsaphor instrument (Pharmacia) with a hexagonal electrode array (9). A program combining three pulse times (30, 60 and 120 s) was used and the size of the DNA fragments was estimated by comparison with concatemerized λ bacteriophage genomes and *Saccharomyces cerevisiae* chromosomes (Bio-Rad).

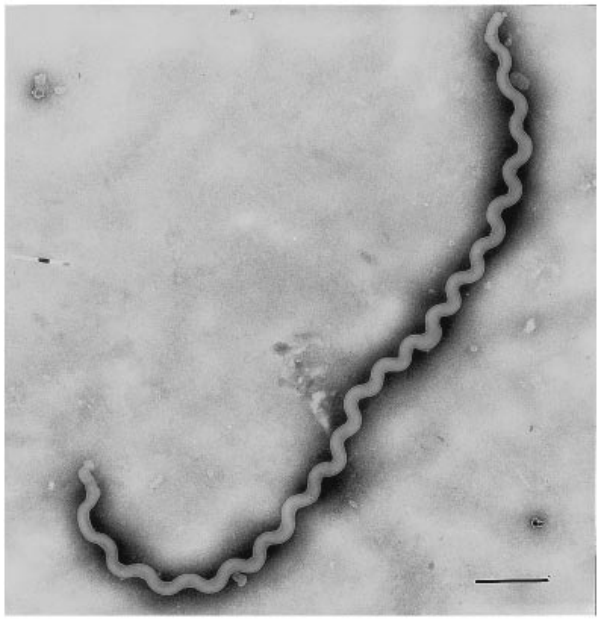

Fig. 1. Electron micrograph of *L. fainei* strain BUT 6^T. Bar, 1 μm.

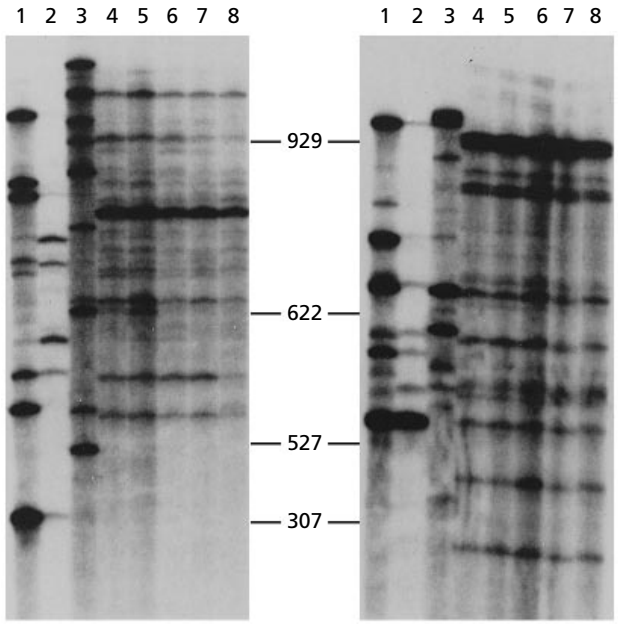

Fig. 2. AP-PCR fingerprints obtained with RSP and KG primers on a 4% polyacrylamide/50% urea sequencing gel. Lanes: 1, *L. interrogans* serovar icterohaemorrhagiae strain RGA^T; 2, *L. interrogans* serovar bratislava strain Jez-bratislava; 3, *L. inadai* serovar lyme strain 10^T; 4–8, *L. fainei* serovar hurstbridge strains BUT 6^T, BUT 8, BKID 6, BKID 7 and WKID. Molecular sizes are indicated in bp.

(iii) MRSP. MRSP of the PCR-amplified leptospiral *rrs* gene was performed as described previously (17, 18) using the primers 16S-11 (5′-GGC TGC AGT CGA CGT TTG ATC CTG GCT CAG-3′) and 16S-1507 (5′-CCA GAT CTG AGC TCA AGG AGG TGA TCC AGC-3′). Briefly, restriction enzyme digestions of PCR-amplified leptospiral *rrs* genes were performed with *Dde*I, *Hha*I, *Nla*IV and *Taq*I and digests were analysed on polyacrylamide native gels autoradiographed for 24–72 h on Kodak X-Omat X-ray film.

(iv) DNA–DNA hybridization. *In vitro* labelling of DNA with tritium-labelled nucleotides and hybridization experiments were performed using the S1 nuclease/TCA method (8) with minor modifications (1). Hybridization reactions were performed in duplicate at an optimal reassociation temperature of 60 °C.

(v) PCR detection of pathogenic signatures on *rrs* (16S rRNA) genes. Aligning the available *rrs* gene sequences for *Leptospira* members (GenBank) by using the vsM program (20), we identified highly conserved regions among pathogenic species that could be used to differentiate saprophytic and pathogenic leptospires. Two corresponding sets of primers including these pathogenic signatures were designed. Set A consisted of primers LP1 (321–371; 5′-GAT TTT TCG GGT AAA GAT TCA TT-3′) and a1190 (1573–1549; 5′-TTG CCC TAG ACA TAA AGG CCA-3′) and allows the amplification of a 1008 bp fragment. The PCR program was: 1 cycle of 3 min at 94 °C, 1·5 min at 61 °C and 2 min at 72 °C; 29 cycles of 1 min at 94 °C, 1·5 min at 61 °C and 2 min at 72 °C; 1 cycle of 1 min at 94 °C, 1·5 min at 61 °C and 10 min at 72 °C. Set B consisted of primers LU (110–131; 5′-CGG CGC GTC TTA AAC ATG-3′) and rLP (681–644; 5′-ACC ATC ATC ACA TYG CTG C-3′) and its PCR product is of 420 bp. The PCR program was the same except that a 56 °C annealing temperature was used. The specificity of these primers had previously been confirmed on the reference strains of the validated pathogenic and saprophytic species of *Leptospira* (P. Perolat & G. Baranton, unpublished data).

(vi) 16S rRNA gene sequencing. BUT 6^T genomic DNA was used as a template for PCR using primers designed to correspond to the sequence of two highly conserved regions within the bacterial 16S rRNA gene. The primers used were 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). The conditions used for amplification were 30 cycles with the profile 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C. Amplified DNA was ligated into pBluescript II KS (Stratagene), which had been first digested with *Eco*RV and modified using *Taq* DNA polymerase to add a single T at the 3′ end (15). Transformants in *Escherichia coli* DH5α containing plasmids with inserts of the expected size were selected and one, pLBA471, was selected for further analysis. The nucleotide sequence of the insert was determined using the Prism dye-terminator system (ABI) on an automated sequencer (model 373; ABI).

RESULTS

Phenotypic characterization

(i) Light and electron microscopy. The strains showed morphology and motility, under dark-field microscopy, that was characteristic of *Leptospira*. Fig. 1 shows an electron micrograph of strain BUT 6^T. Bacterial cells were approximately 12 μm long and 0·2 μm in diameter, with the typical helical morphology characteristic of *Leptospira*.

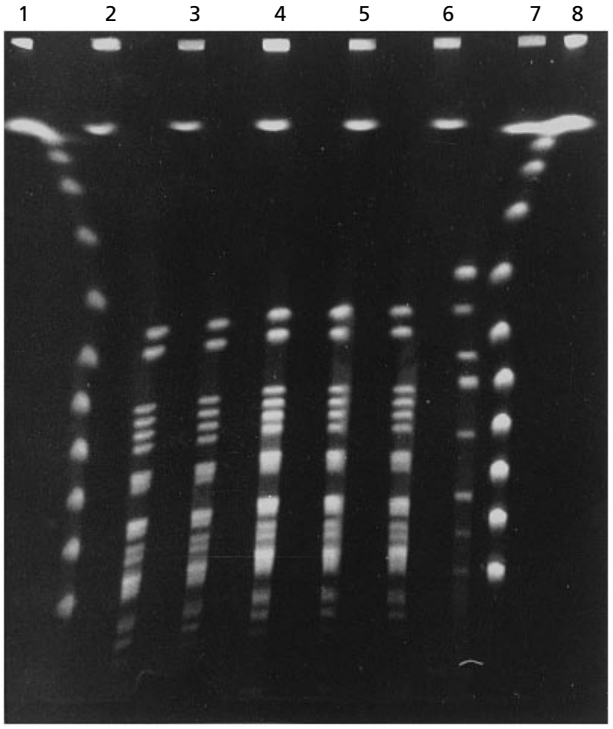

Fig. 3. PFGE of *Not*I restriction fragments from hurstbridge strains. Lanes 1 and 8 are size markers (λ); lanes 2–6 are hurstbridge strain isolates BUT 6^T, BUT 8, BKID 6, BKID 7 and WKID; lane 7, *L. interrogans* serovar icterohaemorrhagiae strain RGA^T. The digestion products were separated at 150 V for 40 h in 1% agarose/0·5 × Tris-borate-EDTA. The three pulse times were 30 s 13 h; 60 s 13 h; 120 s 14 h.

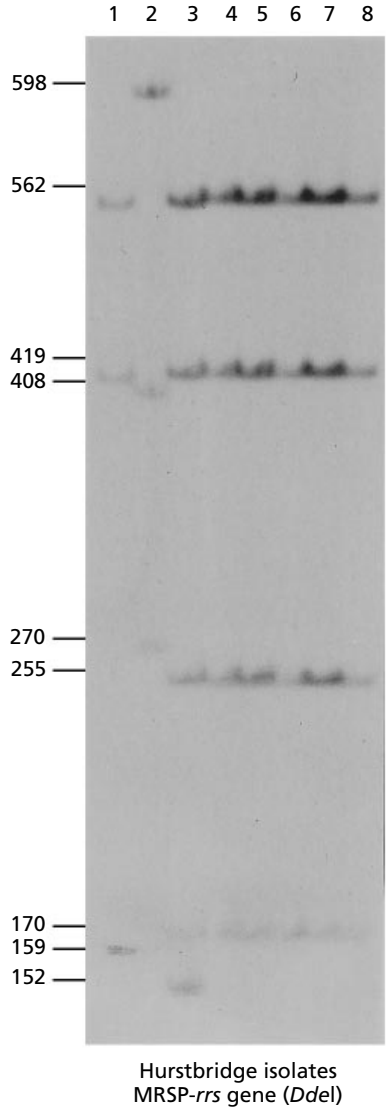

Fig. 4. Autoradiogram of *Dde*I restriction digests of PCR-amplified 16S rRNA genes. Lanes: 1, *L. interrogans* serovar icterohaemorrhagiae strain RGA^T; 2, *L. biflexa* serovar patoc strain Patoc I^T; 3, *L. inadai* serovar lyme strain 10^T; 4–8, *L. fainei* serovar hurstbridge strains BUT 6^T, BUT 8, BKID 6, BKID 7 and WKID. Molecular sizes are indicated in bp.

(ii) Growth characteristics. Hurstbridge strains were able to grow at 13 °C but the density of cells was less than that reached by the saprophytic reference strain Patoc I^T and similar to that of strain 10^T (the reference strain of *L. inadai*). Growth was partially inhibited at 30 °C in the presence of 8-azaguanine. According to these reference tests (11), hurstbridge strains showed growth characteristics intermediate between those of pathogenic and saprophytic *Leptospira* strains.

(iii) Serotyping. No agglutination of hurstbridge strains was observed with any of the 26 reference antisera (7, 10). Rabbit anti-hurstbridge sera (with titres of at least 25600) did not agglutinate any of the 26 reference strains at the recommended dilution of 1/400. There was some low-level cross-agglutination of representatives of Canicola (titre 200), Icterohaemorrhagiae (50) and Semaranga (100) serogroups. Based on these results, and according to SCT recommendations, hurstbridge strains should be considered as belonging to a new serogroup designated Hurstbridge, comprising the serovar hurstbridge.

Genetic characterization

(i) AP-PCR. Characteristic AP-PCR leptospiral fingerprints were obtained with three (KG, KF and RSP) of the nine primers tested. These three primers had previously been demonstrated to produce the most useful patterns for *Leptospira* (13, 17) and representative fingerprints are shown in Fig. 2. The size of the amplified products ranged from 300 to 1000 bp. The patterns of the five hurstbridge strains were identical for a given primer. In addition, no products characteristic of the recognized *Leptospira* species (14, 17, 18) were observed.

(ii) PFGE. The *Not*I DNA restriction patterns of the five hurstbridge strains analysed by PFGE were identical (Fig. 3) and different from those of each of the pathogenic reference strains (9). The size of restriction

Table 1. DNA relatedness of *L. fainei* strain BUT 6^T with *Leptospira* species and *Turneria parva* reference strains (19, 24)

Values are the percentage relative association with labelled DNA from *L. fainei* BUT 6^T, with the percentage relative association with labelled DNA from the corresponding species reference strain in parentheses. T, Type strain.

Species	Relative association (%) with strain BUT 6^T
L. fainei	
BUT 6^T	100·0
WKID	100·0 (100·0)
L. interrogans RGA^T	0·0 (0·0)
L. borgpetersenii ATCC 43292^T	0·0 (9·0)
L. inadai 10^T	34·0 (36·0)
L. kirschneri 3522C^T	2·5 (0·0)
L. meyeri ATCC 43287^T	1·0 (4·5)
L. noguchii ATCC 43288^T	0·0 (7·0)
L. santarosai ATCC 43286^T	1·5 (0·0)
L. weilii ATCC 43285^T	0·0 (4·0)
L. biflexa Patoc I^T	0·0 (0·0)
L. wolbachii ATCC 43284^T	2·0 (1·5)
Turneria parva^T	0·0 (0·0)

fragments ranged from 50 to 1300 kb and a quadruplet in the 200–250 kb zone was observed only in the hurstbridge strains.

(iii) MRSP. As demonstrated previously (18), the primers 16S-11 and 16S-1507 amplified a conserved portion of the *Leptospira* 16S rRNA gene. PCR amplification of the *rrs* gene of each of the hurstbridge strains produced one amplicon of the expected size (1·5 kb). Restriction digestions were performed with *Dde*I (Fig. 4), *Hha*I, *Hinf*I and *Taq*I to investigate the presence of polymorphic restriction sites (18). Among the discriminating restriction sites tested with these four enzymes, the following were absent: *Dde*I in positions 80, 121 and 216; *Hha*I in positions 222 and 640; *Hinf*I in positions 198 and 974; and *Taq*I in position 826. Furthermore, an additional *Taq*I restriction site was present in position 792. The restriction pattern of the 16S rRNA gene (*Dde*I 80−, 121−, 216−; *Hha*I 222−, 640−; *Hinf*I 198−, 974−; *Taq*I 792+, 826−) was identical for each hurstbridge strain, and unrelated to the species-specific MRSP patterns previously established (17, 18). Therefore the hurstbridge strains appeared to represent a new species of *Leptospira*.

(iv) DNA–DNA hybridization. As all five hurstbridge strains appeared identical when tested by AP-PCR and PFGE, DNA–DNA hybridizations were performed between strain BUT 6^T, designated the reference strain for serogroup Hurstbridge and serovar hurstbridge, and pathogenic and saprophytic reference strains (Table 1). To confirm the homogeneity of hurstbridge isolates, DNA–DNA hybridizations were also performed between BUT 6^T and WKID isolates, respectively originated from two distant pig herds, in New South Wales and Victoria (Table 1). The self-DNA binding was 89 and 83·7% for strains BUT 6^T and WKID, respectively.

There was no significant relatedness with any pathogenic or saprophytic species and the least distant was *L. inadai* (34 and 36% in the reciprocal tests). The homogeneity of the hurstbridge strains was confirmed by 100% relative association between BUT 6^T and WKID. As the current concept of a bacterial species is one that would include strains with at least 70% DNA relatedness (23), it was concluded that the hurstbridge strains belong to a new species of *Leptospira*.

(v) PCR detection of pathogenic signatures on 16S rRNA genes. PCR assays on hurstbridge strains, using the

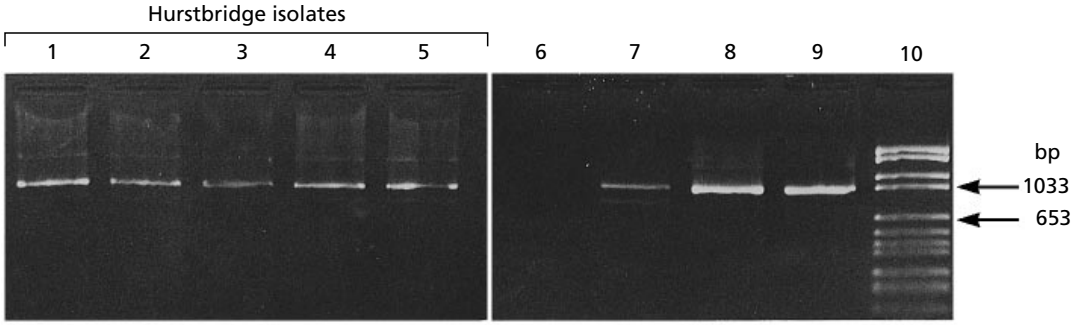

Fig. 5. PCR detection of the leptospiral pathogenic signature on the 16S rRNA gene. The amplification of a 1008 bp 16S rRNA gene fragment was done with the primers LP1/a1190. A 2% Nusieve 3:1 agarose gel stained with ethidium bromide is shown. Lanes: 1–5, *L. fainei* serovar hurstbridge strains WKID, BKID 7, BKID 6, BUT 8 and BUT 6^T; 6, *L. biflexa* serovar patoc strain Patoc I^T; 7, *L. inadai* serovar lyme strain 10^T; 8, *L. interrogans* serovar bratislava strain Jez-bratislava; 9, *L. interrogans* serovar icterohaemorrhagiae strain RGA^T; 10, molecular mass marker (pBR328 DNA digested with *Bgl*I + *Hin*fI).

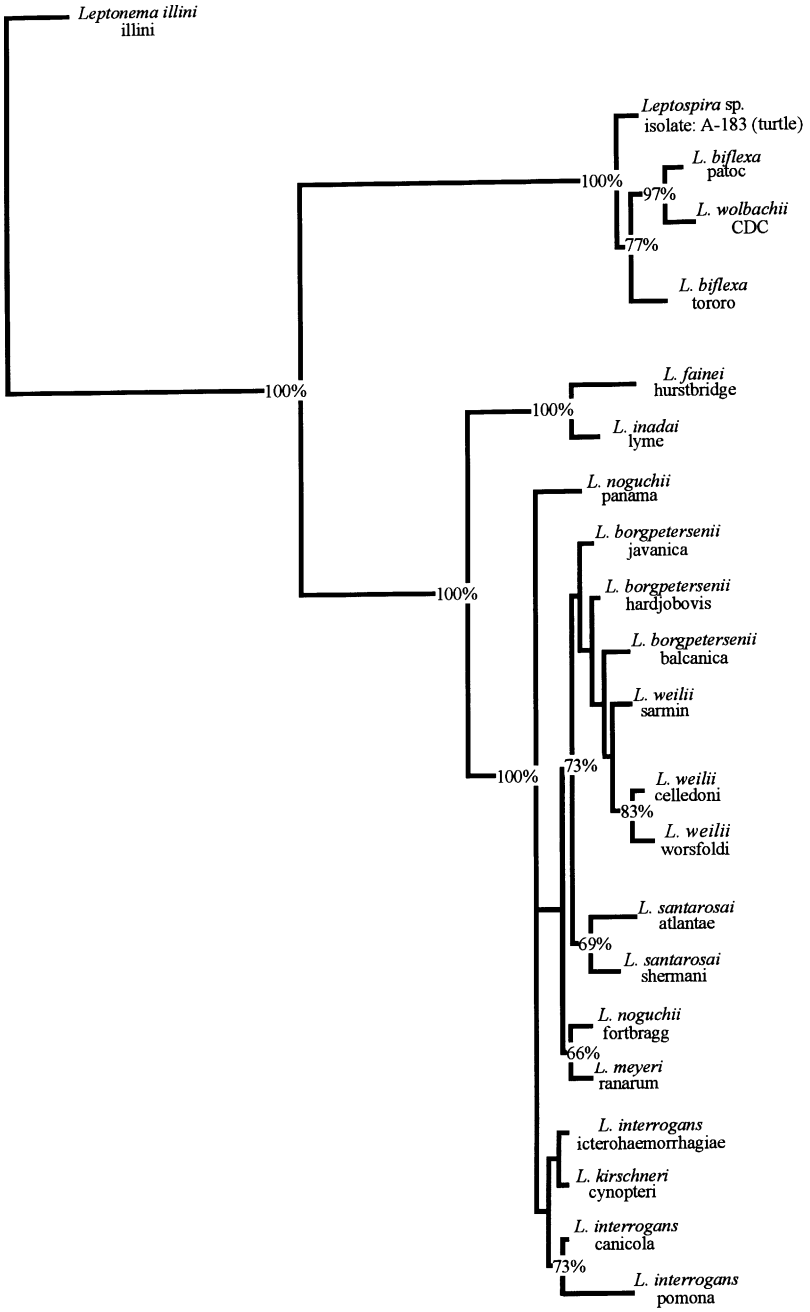

Fig. 6. Tree obtained by parsimony estimation using an alignment of leptospiral 16S rRNA gene sequences, whose accession numbers are listed below. Parsimony analysis was implemented using PAUP (Sinaur Associates). The reliability of branching was assessed using bootstrapping analysis; support for some key nodes is indicated as a percentage. *Leptonema illini* is used as a functional outgroup for showing the root of the tree. The tree shown is one of the six most parsimonious trees found. The branch lengths are scaled in proportion to estimated extent of change in each of the branches using the default settings of PAUP. The total number of steps in the tree is 687. GenBank accession numbers: *L. biflexa* serovar patoc strain Patoc I^T, Z12821; *L. biflexa* serovar tororo strain Tororo, Z26970; *L. borgpetersenii* serovar balcanica strain 1627 Burgas, U12669; *L. borgpetersenii* serovar hardjo type hardjobovis strain Sponselee, U12670; *L. borgpetersenii* serovar javanica strain VB 46^T, Z21630; *L. fainei* serovar hurstbridge strain BUT 6^T, U60594; *L. inadai* serovar lyme strain 10^T, Z21634; *L. interrogans* serovar canicola strain Hond Utrecht IV, X17547; *L. interrogans* serovar icterohaemorrhagiae strain RGA^T, Z12817; *L. interrogans* serovar pomona strain Pomona, M71241; *L. kirschneri* serovar cynopteri strain 3522 C^T, Z21628; *L. meyeri* serovar ranarum strain ICF^T, Z21648; *L. noguchii* serovar fortbragg strain Fort Bragg, U12671; *L. noguchii* serovar panama strain CZ 214^T, Z21635; *L. santarosai* serovar atlantae strain LT 81, U12672; *L. santarosai* serovar shermani strain 1342 K^T, Z21649; *L. weilii* serovar celledoni strain Celledoni^T, Z21637; *L. weilii* serovar sarmin strain Sarmin, U12673; *L. weilii* serovar worsfoldi strain Worsfold, U12677; *L. wolbachii* serovar codice strain CDC^T, Z21638; *Leptonema illini* serovar illini strain 3055^T, Z21632; *Leptospira* sp. turtle isolate, M88721.

two pairs of primers (LP1/a1190 and LU/rLP) specific for pathogenic *Leptospira* 16S rRNA genes, produced products of the expected size: of 1008 bp (Fig. 5) and 420 bp (data not shown), respectively.

(vi) 16S rRNA gene sequencing. A sequence of 1480 nucleotides was determined in the BUT 6^T 16S rRNA gene sequence, corresponding to positions 28–1491 in the *E. coli* numbering system. The phylogenetic tree, including the available sequences of all the known species of *Leptospira*, was inferred using maximum-parsimony analysis (22) and is shown in Fig. 6. Strain BUT 6^T grouped with *L. inadai* and both branched in an intermediate clade close to the clade comprising the pathogenic *Leptospira*. Similar trees were inferred using the unweighted pair group method with averages and the neighbour-joining method (21) (data not shown).

DISCUSSION

The five bacterial strains recovered from kidneys or uteri of sows from two geographically distant

Australian herds had the growth and morphological characteristics of *Leptospira*. They were serologically unrelated to the recognized *Leptospira* serogroups and belong to a new serogroup designated Hurstbridge comprising the serovar hurstbridge.

Initial experiments were used to investigate whether the hurstbridge strains belonged to an existing leptospiral species. Using molecular typing methods (PFGE and AP-PCR fingerprinting), previously demonstrated as suitable for studying the genomic diversity of *Leptospira*, we showed that these strains appeared to be identical. MRSPs in the 16S rRNA gene were different from those in the database of reference strains of *Leptospira* species (19). DNA–DNA hybridizations confirmed the singularity of these isolates when they were compared with the reference strains for existing species (19, 24), leading to the delineation of a new species. Phylogenetic analysis based on 16S rRNA sequences showed that *L. fainei* together with *L. inadai* formed a clade distinct from those containing either pathogenic or saprophytic leptospires.

Description of *Leptospira fainei* sp. nov.

Leptospira fainei (fain′e.i. N.L. gen. n. *fainei* to honour Solomon Faine, Australian medical microbiologist who made definitive contributions to the knowledge of the physiopathology and epidemiology of leptospirosis).

L. fainei grows in EMJH medium (5) at 13 and 30 °C. Under light and electron microscopy, *L. fainei* presents morphology and motility characteristic of the genus *Leptospira* (11). Growth at 30 °C is partially inhibited by 8-azaguanine (225 μg ml^{-1}). *L. fainei* comprises one single serogroup, Hurstbridge, which is not shared with the other *Leptospira* species, identified by cross-agglutination test according to the recommendations of the SCT (10): reference Hurstbridge antiserum only agglutinates, at a significant level ($>1:400$), leptospiral isolates belonging to the Hurstbridge serogroup. A unique serovar, hurstbridge, is included in this serogroup. *L. fainei* is characterized by its specific restriction map of the *rrs* (16S rRNA) gene: *Dde*I 80−, 121−, 216−; *Hha*I 222−, 640−; *Hinf*I 198−, 974−; *Taq*I 792 +, 826−. *Taq*I 792+ is a species-specific restriction site. *L. fainei* was isolated from genito-urinary tracts of pigs and is serologically suspected to circulate in cattle (3). The type strain BUT 6^T has all the properties given for the species. It was isolated from the uterus of a sow in New South Wales (Australia) by Chappel *et al.* (3).

ACKNOWLEDGEMENTS

We thank Mr Chris Schroen for performing the electron microscopy and Mrs Elisabeth Bellenger, Irene Lecuyer and Vicki Vallance for technical assistance. Financial support was provided by the Pig Research and Development Corporation, Australia, the International Network of Institutes Pasteur, the National Health and Medical Research Council, Australia, and the French Ministry of Foreign Affairs.

REFERENCES

1. **Baranton, G., Postic, D., Saint Girons, I., Boerlin, P., Pifaretti, J. C., Assous, M. & Grimont, P. A. D. (1992).** Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int J Syst Bacteriol* **42**, 378–383.
2. **Brenner, D. J., McWhorter, A. C., Leete Knutson, J. K. & Steigerwalt, A. G. (1982).** *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J Clin Microbiol* **15**, 1133–1140.
3. **Chappel, R. J., Billinghurst, M. L., Adler, B., Bulach, D. M., Khalik, D., Merien, F., Serrano, M. S., Wilson, R. J. & Perolat, P. (1996).** Isolation from pigs of a pathogenic leptospire of a new genospecies and serogroup (serovar hurstbridge), and a serological study of its distribution and possible veterinary significance. *Proceedings of the First Meeting of the International Leptospirosis Society*, Nantes.
4. **Dikken, H. & Kmety, E. (1978).** Serological typing methods of leptospires. *Methods Microbiol* **11**, 259–307.
5. **Ellinghausen, H. C. & McCullough, W. G. (1965).** Nutrition of *Leptospira* pomona and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. *Am J Vet Res* **26**, 45–51.
6. **Ellis, W. A., Montgomery, J. M. & Thiermann, A. B. (1991).** Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the Australis serogroup of *Leptospira interrogans*. *J Clin Microbiol* **29**, 957–961.
7. **Faine, S. (1982).** Guidelines for leptospirosis control. Geneva: World Health Organization.
8. **Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C. & Lemelin, M. (1980).** Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr Microbiol* **4**, 326–330.
9. **Herrmann, J. L., Bellenger, E., Perolat, P., Baranton, G. & Saint Girons, I. (1992).** Pulsed-field gel electrophoresis of *Not*I digests of leptospiral DNA: a new rapid method of serovar identification. *J Clin Microbiol* **30**, 1696–1702.
10. **International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira* (1987).** Minutes of the Meeting, 5 and 6 September 1986, Manchester, UK. *Int J Syst Bacteriol* **37**, 472–473.
11. **Johnson, R. C. & Faine, S. (1984).** Family II. Leptospiraceae. Hovind-Hougen 1979, 245^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 62–67. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
12. **Johnson, R. C. & Rogers, P. (1964).** Differentiation of pathogenic and saprophytic leptospires with 8-azaguanine. *J Bacteriol* **88**, 1618–1623.
13. **Kmety, E. & Dikken, H. (1993).** *Classification of the Species Leptospira interrogans and History of its Serovars*. Groningen: University Press.
14. **Letocart, M., Baranton, G. & Perolat, P. (1997).** Rapid identification of pathogenic *Leptospira* species (*L. interrogans*, *L. borgpetersenii*, and *L. kirschneri*) with species-specific probes produced by arbitrarily primed PCR. *J Clin Microbiol* **35**, 248–253.

15. **Marchuk, D., Drumm, M., Saulino, A. & Collins, F. S. (1991).** Construction of T-vectors, a rapid general system for the direct cloning of unmodified DNA products. *Nucleic Acids Res* **19**, 1154.

16. **Perolat, P., Lecuyer, I., Postic, D. & Baranton, G. (1993).** Diversity of ribosomal DNA fingerprints of *Leptospira* serovars provides a database for subtyping and species assignation. *Res Microbiol* **144**, 5–15.

17. **Perolat, P., Merien, F., Ellis, W. A. & Baranton, G. (1994).** Characterization of *Leptospira* isolates from serovar hardjo by ribotyping, arbitrarily-primed PCR, and mapped restriction site polymorphisms. *J Clin Microbiol* **32**, 1949–1957.

18. **Ralph, D., McClelland, M., Welsh, J., Baranton, G. & Perolat, P. (1993).** *Leptospira* species categorized by arbitrarily-primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *J Bacteriol* **175**, 973–981.

19. **Ramadass, P., Jarvis, B. D. W., Corner, R. J., Penny, D. & Marshall, R. B. (1992).** Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. *Int J Syst Bacteriol* **42**, 215–219.

20. **Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Boussemart, O., Gauthier, M. & Christen, R. (1994).** Phylogenetic analysis and assessment of the genera *Vibrio*, *Protobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* **44**, 416–426.

21. **Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

22. **Swofford, D. L. (1991).** PAUP: phylogenetic analysis using parsimony, version 3.0. Illinois Natural History Survey, Champaign, IL, USA.

23. **Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 407–415.

24. **Yasuda, P. H., Steigerwalt, A. G., Sulzer, K. R., Kaufmann, A. F., Rogers, F. & Brenner, D. J. (1987).** Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. *Int J Syst Bacteriol* **37**, 407–415.

25. **Zuerner, R. L. & Bolin, C. A. (1990).** Nucleic acid probe characterizes *Leptospira interrogans* serovars by restriction fragment length polymorphisms. *Vet Microbiol* **24**, 355–366.