Genetic diversity of *Leptotrichia* and description of *Leptotrichia goodfellowii* sp. nov., *Leptotrichia hofstadii* sp. nov., *Leptotrichia shahii* sp. nov. and *Leptotrichia wadei* sp. nov.

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Sixty strains of Gram-negative, anaerobic, rod-shaped bacteria from human sources initially assigned to *Leptotrichia buccalis* ($n=58$) and '*Leptotrichia pseudobuccalis*' ($n=2$) have been subjected to polyphasic taxonomy. Full-length 16S rDNA sequencing, DNA–DNA hybridization, RAPD, SDS-PAGE of whole-cell proteins, cellular fatty acid analysis and enzymic/biochemical tests supported the establishment of four novel *Leptotrichia* species from this collection, *Leptotrichia goodfellowii* sp. nov. (type strain LB 57^T=CCUG 32286^T=CIP 107915^T), *Leptotrichia hofstadii* sp. nov. (type strain LB 23^T=CCUG 47504^T=CIP 107917^T), *Leptotrichia shahii* sp. nov. (type strain LB 37^T=CCUG 47503^T=CIP 107916^T) and *Leptotrichia wadei* sp. nov. (type strain LB 16^T=CCUG 47505^T=CIP 107918^T). Light and electron microscopy showed that the four novel species were Gram-negative, non-spore-forming and non-motile rods. *L. goodfellowii* produced arginine dihydrolase, β -galactosidase, *N*-acetyl- β -glucosaminidase, arginine arylamidase, leucine arylamidase and histidine arylamidase. *L. shahii* produced α -arabinosidase. *L. buccalis* and *L. goodfellowii* fermented mannose and were β -galactosidase-6-phosphate positive. *L. goodfellowii*, *L. hofstadii* and *L. wadei* were β -haemolytic. *L. buccalis* fermented raffinose. With *L. buccalis*, *L. goodfellowii* showed 3.8–5.5% DNA–DNA relatedness, *L. shahii* showed 24.5–34.1% relatedness, *L. hofstadii* showed 27.3–36.3% relatedness and *L. wadei* showed 24.1–35.9% relatedness. 16S rDNA sequencing demonstrated that *L. hofstadii*, *L. shahii*, *L. wadei* and *L. goodfellowii* each formed individual clusters with 97, 96, 94 and 92% similarity, respectively, to *L. buccalis*.

INTRODUCTION

Based on comparative analysis of 16S rRNA sequences, *Leptotrichia* is one of six genera in the family *Fusobacteriaceae* in the phylum *Fusobacteria*. The genus *Leptotrichia* contains *Leptotrichia buccalis*, the type species of the genus

(Hofstad, 1984), and two recently defined species, *Leptotrichia trevisanii* (Tee *et al.*, 2001) and '*Leptotrichia amnionii*' (Shukla *et al.*, 2002). *L. trevisanii* was isolated from the blood of a man with acute myeloid leukaemia (Tee *et al.*, 2001) and '*L. amnionii*' from the amniotic fluid of a woman

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The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *L. hofstadii* LB 23^T, *L. shahii* LB 37^T, *L. wadei* LB 16^T and *L. goodfellowii* LB 57^T are AY029803, AY029806, AY029802 and AY029807.

after intrauterine fetal demise (Shukla *et al.*, 2002). The closely related species '*Leptotrichia sanguinegens*' (Hanff *et al.*, 1995a) (= '*Leptotrichia microbii*'; Hanff *et al.*, 1995b) was recently transferred to a new genus *Sneathia* as *Sneathia sanguinegens* (Collins *et al.*, 2001).

Leptotrichia species are large, fusiform, non-sporulating and non-motile rods with a Gram-negative-like cell wall (Hofstad & Selvig, 1969). The primary habitat of *Leptotrichia* is the human oral cavity, where they are typically found in dental plaque (Hofstad & Olsen, 2004). However, *Leptotrichia* has been also isolated from the normal flora of the periurethral region of healthy girls and the genitalia of women (Moore *et al.*, 1976; Söderberg *et al.*, 1979; Evaldson *et al.*, 1980). *L. buccalis* or *L. buccalis*-like bacteria have occasionally been recovered from blood, mostly in immunocompromised patients with neutropenia and from endocarditis (Reig *et al.*, 1985; Weinberger *et al.*, 1991; Hammann *et al.*, 1993; Messiaen *et al.*, 1996; Vernelen *et al.*, 1996; Patel *et al.*, 1999).

The taxonomy of *Leptotrichia* has been somewhat vague. For example, *L. buccalis* has often been misclassified as species of *Fusobacterium* and *Lactobacillus* (Hamilton & Zahler, 1957). Indeed, even within the genus *Leptotrichia*, there is significant heterogeneity in enzymic/biochemical reactions (Eribe *et al.*, 2002) and in cellular fatty acid content (Hofstad & Jantzen, 1982; Eribe *et al.*, 2002). Furthermore, significant variation among 60 strains of *Leptotrichia* was observed in SDS-PAGE profiles of whole-cell proteins and RAPD patterns of DNA (Eribe & Olsen, 2002). In the present study, these 60 strains were further analysed to examine their full taxonomic and phylogenetic diversity by using full-length 16S rDNA sequencing, DNA–DNA hybridization and colony and cell morphology assessment.

METHODS

Bacteria. The 60 strains of *Leptotrichia* used, with their site of isolation and source, are listed in Table 1. The isolates originated from gingival/periodontal pockets of man, human saliva, dental plaque, supragingival calculus, human synovia, septic arthritis and unknown sources. They fulfilled the novel species requirements for different sources with respect to geography and ecology (Christensen *et al.*, 2001). All isolates were received from well-recognized private or public culture collections in Europe and America where they had been characterized.

Cultivation of bacterial strains. The organisms were inoculated from frozen stock onto Columbia (AppliChem) agar or brain heart infusion (BHI) (AppliChem) agar plates supplemented with 5% human blood, 50 mg haemin ml⁻¹ (Sigma) and 5 mg menadione ml⁻¹ (Sigma). They were cultured anaerobically (90% N₂, 5% H₂ and 5% CO₂) at 37 °C for 2–5 days in evacuation jars (Anoxomat System, WS9000; Mart). After replating the cultures, single colonies were picked and subcultured anaerobically on Columbia blood agar plates at 37 °C for 2–5 days. After culturing, bacterial cells were harvested by centrifugation (11 000 r.p.m.) for 5 min and stored at –20 °C if not used immediately.

DNA extraction for 16S rDNA analysis. DNA was extracted according to the procedure of Popovic *et al.* (1993) with some

modifications (Eribe & Olsen, 2000). Bacterial DNA was resuspended in 100 µl TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and its quality checked spectrophotometrically and by gel electrophoresis.

Amplification of 16S rDNA and purification of PCR products.

The 16S rRNA genes from the examined strains were amplified under standardized conditions with the primers 9F forward and 1541R reverse (Table 2). PCR was performed in thin-walled tubes with a Perkin-Elmer 9700 thermocycler. One microlitre of the diluted (1:5) DNA template was added to a reaction mixture (50 µl final volume) containing 1× *Taq* 2000 reaction buffer, 2.5 mM MgCl₂, 0.8 µM dNTPs, 400 nM of each primer and 1 U *Taq* 2000 polymerase (Stratagene) in buffer containing *Taq*start antibody (Sigma). In a hot-start protocol, samples were preheated at 94 °C for 4 min followed by amplification under the following conditions: denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s and extension for 1.5 min with an additional 1 s for each cycle. A total of 30 cycles was performed, followed by a final elongation step at 72 °C for 15 min. The products of PCR amplification were examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light. Amplified 16S rRNA genes were purified with the Sequenase kit (Amersham Pharmacia) for partial sequencing and on Sephadex G-50 resin columns before full-length sequencing.

16S rDNA sequencing. Purified DNA from PCR was sequenced with an ABI Prism cycle-sequencing kit (BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer). Quarter-dye chemistry was applied with 3.2 µM primers and 1.5 µl PCR products in a final volume of 20 µl. Cycle sequencing was done with an ABI 9700 PCR machine with 25 cycles of denaturation at 96 °C for 10 s and annealing and extension at 60 °C for 4 min. Purified DNA products were dried in a speed vac for 75 min, resuspended in 3.2 µl loading dye and denatured at 98 °C for 2 min. The denatured products were then kept on ice and, finally, 1.5 µl DNA was loaded into the 8% Long-Ranger polyacrylamide gel (FMC Bioproducts). Sequencing reactions were run on an ABI Prism 377 DNA sequencer.

Data analyses of 16S rDNA sequences. First, approximately 410 bases were sequenced to determine the identity or approximate phylogenetic position of the 60 strains. Using six additional sequencing primers (Table 2), full sequences (about 1500 bases) were then obtained for 11 representative strains, some of which represented putative novel species. For identification of the closest relatives, the sequences of the unrecognized strains were compared with the 16S rRNA gene sequences of over 9000 bacteria in Paster and Dewhirst's database and 76 000 sequences in the Ribosomal Data Project (RDP) (Cole *et al.*, 2003). Similarity matrices were corrected for multiple base changes at single positions by the method of Jukes & Cantor (1969). Phylogenetic trees were constructed by the neighbour-joining method of Saitou & Nei (1987). Sequences were aligned using the MegAlign program (DNASTAR) and imported into TREECON, a software package for the Microsoft Windows environment, which was used for the construction and drawing of evolutionary trees (Van de Peer *et al.*, 1996).

Nucleotide sequence accession numbers. The complete 16S rRNA gene sequences of the novel species were deposited in 2001 in GenBank under the accession numbers listed in Fig. 1.

DNA–DNA relatedness assays. The S₁-nuclease procedure for the free-solution reassociation for DNA similarity assays was used for pairwise reactions (Johnson, 1994) with selected strains of *Leptotrichia*. All procedures, including DNA isolation, French pressure cell fragmentation, hybridization and S₁-nuclease assays have been detailed elsewhere (Johnson, 1994). However, rather than labelling

Table 1. Strains originally assigned as *L. buccalis* and '*L. pseudobuccalis*' used in this study

Original assignments were derived from Eribe *et al.* (2002): LB, *L. buccalis*; LPB, '*L. pseudobuccalis*'. Culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; VPI, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA; CCUG, Culture Collection of Göteborg, Göteborg, Sweden. NK, Not known.

Strain	Reference no.	Source and other designation	Site of isolation
LB 1	1-2	Gade Institute, Bergen, Norway	Gingival pocket, periodontitis
LB 2	1-4	Gade Institute, Bergen, Norway	Gingival pocket, periodontitis
LB 3	8-1	Gade Institute, Bergen, Norway	Gingival pocket, periodontitis
LB 4	11-1	Gade Institute, Bergen, Norway	Gingival pocket, periodontitis
LB 5	11-2	Gade Institute, Bergen, Norway	Gingival pocket, periodontitis
LB 6	82A-026	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 7	89A-069	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 8	90-0274	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 9	90-0309	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 10	91A064	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 11	94-0155	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 12	94-0200	K. Bernard, Ottawa, Ontario, Canada	Blood
LB 13 ^T	ATCC 14201 ^T	Gade Institute, Bergen, Norway	Supragingival calculus
LB 14	ES 23-91	R. Hammann, Heidelberg, Germany	Blood
LB 15	L 11	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 16	L 41	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 17	L 42	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 18	L 43	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 19	L 44	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 20	L 45	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 21	L 46	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 22	L 47	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 23	L 48	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 24	L 49	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 25	L 50	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 26	L 51	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 27	L 90	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 28	L 92	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 29	L 101	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 30	L 102	Gade Institute, Bergen, Norway	Healthy person, saliva
LB 31	RYC 29853	M. Reig, Madrid, Spain	Blood
LB 32	RYC 30220	M. Reig, Madrid, Spain	Blood
LB 33	ATCC 19616	Gade Institute, Bergen, Norway	NK
LB 37	N06A-34	VPI, Blacksburg, VA, USA	Gingivitis
LB 47	W16A-16	VPI, Blacksburg, VA, USA	Juvenile periodontitis
LB 48	CCUG 19067B	Danderyd PHL, Danderyd, Sweden	Blood
LB 49	CCUG 19169	M. Wickström, Göteborg, Sweden	Subgingival pocket
LB 50	CCUG 19427	Lund PHL, Lund, Sweden	NK
LB 51	CCUG 21070	Uddevalla PHL, Uddevalla, Sweden	Blood, leukaemia
LB 52	CCUG 23775	PHL, Göteborg, Sweden	Blood
LB 53	CCUG 29716	Ha, Uppsala, Sweden	NK
LB 54	CCUG 30637	Gävle, Sweden	NK
LB 55	CCUG 32116	Gävle PHL, Gävle, Sweden	Synovia, septic arthritis
LB 56	CCUG 32173	PHL, Göteborg, Sweden	Blood
LB 57	CCUG 32286	Heidelberg, Germany	Blood
LB 58	CCUG 33471	PHL, Göteborg, Sweden	Human
LB 59	CCUG 34278	PHL, Göteborg, Sweden	Human
LB 61	CCUG 34631	PHL, Sundsvall, Sweden	Blood
LB 62	CCUG 35982	Karlstad PHL, Karlstad, Sweden	Blood, acute leukaemia
LB 63	CCUG 36771	Uppsala, Sweden	Blood

Table 1. cont.

Strain	Reference no.	Source and other designation	Site of isolation
LB 64	CCUG 36868	Tromsø, Norway	Blood
LB 65	CCUG 36980	Skövde PHL, Skövde, Sweden	Blood
LB 66	CCUG 38157	PHLS, Halmstad, Sweden	Blood, leukaemia
LB 67	CCUG 38343	Lindköping, Sweden	Sputum
LPB 68	CCUG 32006	Sundsvall, Sweden	Blood
LPB 69	CCUG 34137	PHL, Danderyd, Sweden	Blood
LB 70	ATCC 23471	G. Frostell strain 18, Sweden	Oral cavity
LB 71	ATCC 23472	G. Frostell strain 19, Sweden	Oral cavity
LB 76	CCUG 41699	PHLS, Halmstad, Sweden	Blood, pneumonia
LB 77	CCUG 42664	PHL, Karlstad, Sweden	Blood, leukaemia

the probe DNA chemically with ^{125}I , the random primers method was used (Random Primers DNA labelling system; Invitrogen Life Technologies) to label DNA with [α - ^{32}P]dCTP (Perkin Elmer Life Sciences). The probe and target DNAs were reassociated at $66.2 \pm 0.5^\circ\text{C}$ for 24 h for *Leptotrichia* DNA with a mean G+C content of 29.7 mol% (Tee *et al.*, 2001). Values for both homologous and heterologous reassociations were corrected for non-specific heteroduplex formation by control reactions with salmon sperm single-stranded DNA (0% DNA relatedness) and were less than 10%. Each reaction was repeated at least three times. The mean of all reactions was reported as percentage DNA–DNA relatedness. DNA from *Erwinia amylovora* ATCC 29780 (EA 110) was used as the outgroup.

Morphological studies. Cellular morphology of the novel species was determined by examining cells grown anaerobically at 37°C on Columbia or BHI blood agar plates. Light microscopy (LM) and transmission (TEM) and scanning (SEM) electron microscopy were used for characterization of cell morphology and ultrastructure. With LM, bacterial cells were also checked for motility in wet mounts and for Gram-staining.

RESULTS AND DISCUSSION

16S rDNA sequencing

Based on comparisons of nearly complete 16S rDNA sequences, the phylogenetic relationships of the 60 *Leptotrichia* isolates revealed that there were four putative novel species (Fig. 1), namely *Leptotrichia goodfellowii* sp. nov.

(LB 57^T), *Leptotrichia hofstadii* sp. nov. (LB 23^T), *Leptotrichia shahii* sp. nov. (LB 37^T) and *Leptotrichia wadei* sp. nov. (LB 16^T). Sequence database searches confirmed that these species were related, but clearly different from each other and from *L. buccalis*. Strains LB 06 and LB 11, which were essentially identical (Fig. 1), had 99.6 and 99.7% sequence similarity to the recently proposed *L. trevisanii* (Tee *et al.*, 2001).

L. wadei LB 16^T, from the saliva of a healthy man, clustered with a previously described strain, A39FD, that was isolated from subgingival plaque (Fig. 1). LB 16^T had 94% sequence similarity to *L. buccalis*.

The type strains of *L. hofstadii* (LB 23^T), *L. shahii* (LB 37^T) and *L. goodfellowii* (LB 57^T) each formed individual clusters that respectively showed 97, 96 and 92% sequence similarity to *L. buccalis*. *L. hofstadii* LB 23^T, isolated from the saliva of a healthy man, clustered with a previously described strain (FAC5) that was isolated from dental plaque. *L. shahii* LB 37^T, from dental plaque of a gingivitis patient, clustered with DE081, a previously described phylotype from dental plaque. *L. goodfellowii* LB 57^T was isolated from human blood. *Leptotrichia* isolates have repeatedly been recovered from cases of bacteraemia (Reig *et al.*, 1985; Weinberger *et al.*, 1991; Schwartz *et al.*, 1995; Patel *et al.*, 1999; Tee *et al.*, 2001), suggesting that they may be involved in

Table 2. Primers used for amplification and sequencing of 16S rRNA genes

Primer	Orientation	Function	Position (bp)*	Sequence (5'–3')
9F	Forward	PCR	9–27	GAGTTTGATYCTGGCTCAG
338F	Forward	Sequencing	339–358	CTCCTACGGGAGGCAGCAGT
531R	Reverse	Sequencing	509–527	TACCGCGGCTGCTGGCAC
789F	Forward	Sequencing	789–806	TAGATACCCYGGTAGTCC
926R	Reverse	Sequencing	896–915	CCGTCWATTCMTTGTGATTT
1099F	Forward	Sequencing	1099–1113	GCAACGAGCGCAACC
1193R	Reverse	Sequencing	1175–1194	ACGTCATCCCCACCTTCCTC
1541R	Reverse	PCR	1526–1541	GAAGGAGGTGWTCCARCCGCA

*Numbered relative to *Escherichia coli* (Woese *et al.*, 1983).

Fig. 1. Phylogenetic tree of full-length 16S rRNA gene sequences showing positions of *L. goodfellowii* sp. nov. LB 57^T, *L. hofstadii* sp. nov. LB 23^T, *L. shahii* sp. nov. LB 37^T and *L. wadei* sp. nov. LB 16^T. Strains LB 6 and LB 11 have almost identical sequences (99.6 and 97.0%, respectively) to that of *L. trevisanii* LT^T. Bar, 5% difference in nucleotide sequences as estimated using the correction of Jukes & Cantor (1969). Distances are determined by measuring the lengths of the horizontal lines connecting two sequences. Numbers at branching points are bootstrap percentages. GenBank accession numbers are given.

non-oral infections of the body. For example, *Leptotrichia* has been associated with endocarditis (Duperval *et al.*, 1984; Hammann *et al.*, 1993), liver abscess (Messiaen *et al.*, 1996) and neutropenia in immunocompromised hosts (Reig *et al.*, 1985; Baquero *et al.*, 1990; Patel *et al.*, 1999).

Close relatives of *L. goodfellowii* were phylotypes EI022 and EI013 (Fig. 1), which had been derived from the subgingival pocket of a healthy subject. Each of these phylotypes likely represents additional species.

Clone DA069 (Fig. 1) is essentially identical to a novel *Leptotrichia*-like isolate (accession no. AF182944) from a neutropenic patient (Patel *et al.*, 1999), indicating that the source of this species is indeed the oral cavity.

'*L. sanguinegens*' (= '*L. microbii*') (Hanff *et al.*, 1995a, b) has recently been reclassified as *Sneathia sanguinegens* (Collins *et al.*, 2001). Our data (Fig. 1) suggest that '*L. amnionii*' would be better assigned to the genus *Sneathia* than to *Leptotrichia*.

DNA–DNA relatedness

Phylogenetic relationships of the putative novel species were confirmed by using DNA–DNA relatedness. It has been suggested that differences of >1–2% between RNA sequences are sufficient to characterize bacteria as taxonomically different. In addition, those organisms that differ by more than 3% in sequence comparisons rarely display more than 60% DNA–DNA relatedness (Stackebrandt & Goebel, 1994). The differences between the sequences of the designated novel species and that of *L. buccalis* were greater than 3%.

The S₁-nuclease method of free-solution reassociation for DNA similarity assays has been recommended for definition

of bacterial species (Steigerwalt *et al.*, 1976; Johnson, 1994; Stackebrandt & Goebel, 1994). Six relatedness groups within the genus *Leptotrichia* were defined (Table 3). These relatedness groups correlated well with phylogenetic clustering (Fig. 1). The phylogenetic tree (not shown) generated from the DNA–DNA relatedness results of the seven labelled (LB 6, LB 13^T, LB 16, LB 23, LB 37^T, LB 57^T and *L. trevisanii* LT^T) and two unlabelled (LB 11 and EA 110) strains was divided into seven clusters (Table 3). One cluster consisted of LB 6, LB 11 and *L. trevisanii* LT^T. 16S rRNA gene sequencing had grouped LB 6 and LB 11 together (Fig. 1) and LB 16 and strain A39FD. DNA–DNA relatedness of more than 70% is a key definition for strains belonging to the same species (Johnson, 1973; Steigerwalt *et al.*, 1976; Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Strains LB 6 and LB 11 showed more than 70% relatedness with *L. trevisanii* (Table 3) and should be considered strains of this species. There was complete agreement between the 16S rRNA gene sequences and DNA–DNA relatedness results for strains LB 23^T, LB 37^T and LB 57^T. Based on these results, separate species designation of the putative novel species is warranted. The type strain of *L. buccalis* constituted a cluster by itself and *E. amylovora* formed an outgroup, as expected.

The phylogenetic diversity supported previous phenotypic and genotypic data from the same isolates examined for enzymic/biochemical reactions, cellular fatty acid content, SDS-PAGE patterns of whole-cell proteins and RAPD profiles (Eribe *et al.*, 2002; Eribe & Olsen, 2002). Key characteristics that differentiate these species are listed in Table 4. Dendrograms based on 70% similarity of fingerprints from SDS-PAGE of whole-cell protein and RAPD with primers OPA 3 and OPA 5 (Dendron computer-assisted gel analysis) showed that the novel species were clearly different from each other and from *L. buccalis* (results

Table 3. Percentage DNA–DNA relatedness among *Leptotrichia* species

Species-level ($\geq 70\%$) groupings are boxed. *E. amylovora* was included as an outgroup.

Source of labelled DNA	DNA–DNA relatedness with unlabelled DNA from:						
	1	2	3	5	6	7	8
1. <i>L. buccalis</i> LB13 ^T	100·0	30·9	39·1	35·9	27·3	34·1	3·8
2. <i>L. trevisanii</i> LT ^T	42·8	100·0	91·4	38·7	36·3	37·4	4·1
3. Strain LB 6	40·3	82·9	100·0	45·7	45·4	40·5	5·2
4. Strain LB 11	36·4	69·5	71·3	36·5	38·4	33·0	3·1
5. <i>L. wadei</i> LB 16 ^T	24·1	20·9	21·6	100·0	25·4	56·7	2·5
6. <i>L. hofstadii</i> LB 23 ^T	36·3	34·6	32·6	35·9	100·0	39·0	4·6
7. <i>L. shahii</i> LB 37 ^T	24·5	25·0	35·4	52·6	24·7	100·0	2·7
8. <i>L. goodfellowii</i> LB 57 ^T	5·5	7·5	9·3	9·3	8·7	10·8	100·0
9. <i>Erwinia amylovora</i> EA 110	−4·3	2·2	2·9	2·8	−0·6	2·3	1·6

Table 4. Salient characteristics of *Leptotrichia* species

Strains: 1, *L. buccalis* LB 13^T; 2, *L. goodfellowii* LB 57^T; 3, *L. hofstadii* LB 23^T; 4, *L. shahii* LB 37^T; 5, *L. wadei* LB 16^T. Data were partly derived from Eribe *et al.* (2002). All strains show optimum growth at 37 °C and are positive for catalase and aesculin. Predominant cellular fatty acids were detected with the Sherlock system; values are percentages of total fatty acids. DMA, Dimethyl acetal; ECL, equivalent chain-length.

Characteristic	1	2	3	4	5
Number of strains	26	3	7	1	11
Number of clones	0	1	0	1	0
Growth at:					
25 °C	+	−	−	+	−
42 °C	+	−	−	−	−
Haemolysis of human full blood	−	+	+	−	+
ID 32 A results:					
Arginine dihydrolase	−	+	−	−	−
α -Galactosidase	+	−	−	−	−
β -Galactosidase	−	+	−	−	−
β -Galactosidase-6-phosphate	+	−	+	−	−
α -Glucosidase	+	−	+	+	+
β -Glucosidase	+	+	+	−	+
α -Arabinosidase	−	−	−	+	−
N-Acetyl- β -glucosaminidase	−	+	−	−	−
Mannose fermentation	+	−	+	−	−
Raffinose fermentation	+	−	−	−	−
Alkaline phosphatase	+	+	+	−	−
Arginine arylamidase	−	+	−	−	−
Leucine arylamidase	−	+	−	−	−
Tyrosine arylamidase	+	−	−	−	−
Histidine arylamidase	−	+	−	−	−
Profile	4616400100	2111412001	0612400000	0420000000	0410000000
Saturated straight-chain fatty acids:					
14:0	10	14	6	9	7
16:0	39	41	48	32	45
Unsaturated straight-chain fatty acids:					
18:1-c11/t9/t6 or unknown with ECL of 17·834	42	28	27	36	24
Hydroxy fatty acids:					
3-OH-14:0 or 15:0 DMA	7	9	9	5	8

not shown). Particularly, DNA–DNA relatedness and SDS-PAGE showed good congruence that supported Vauterin *et al.* (1993), emphasizing the fitness of protein electrophoresis in bacterial classification. Also, the 16S rRNA phylogenetic tree corresponded well with the SDS-PAGE dendrogram. Thus, our studies demonstrate the value of polyphasic taxonomy for establishing novel bacterial species (Vandamme *et al.*, 1996).

Morphological studies using LM, SEM and TEM showed that the four novel species had several features in common. They were Gram-negative, non-spore-forming and non-motile rods. Cells were arranged in pairs, some slightly curved, others in chains joined by flattened ends. Electron micrographs of ultrathin sections revealed elongated cells with a cell-wall architecture typical of Gram-negative bacteria. A double plasma membrane layer, a single electron-dense (intermediate) layer, a double outer layer and scale-like protrusions were observed (Fig. 2a, c, e and g). A cross-section of the cell showed storage granules. SEM of *L. goodfellowii* demonstrated short rods, varying in length from 2 to 4 µm and from 0.3 to 0.6 µm in width, with one end tapered (Fig. 2b). SEM of *L. hofstadii* revealed long rods, 7.5–15 µm long and 0.5–0.9 µm wide, with one end tapered (Fig. 2d). *L. shahii* exhibited long rods, 7.5–17.5 µm long and 0.5–0.9 µm wide, with one end tapered (Fig. 2f), and *L. wadei* had short rods, 2.5–10 µm long and 0.3–0.6 µm wide, with one end tapered (Fig. 2h).

Based on the results from enzymic/biochemical reactions, cellular fatty acid analysis, SDS-PAGE of whole-cell proteins and RAPD (Eribe *et al.*, 2002; Eribe & Olsen, 2002) and those of the current study related to 16S rDNA sequencing and DNA–DNA relatedness, four previously unrecognized *Leptotrichia* species could be established. However, it is likely that additional species of *Leptotrichia* are present in the oral cavity. In sequence analysis of clones of 16S rDNA genes amplified directly from sites in the oral cavity (Paster *et al.*, 2001; Becker *et al.*, 2002), several not-yet-cultivated species were detected that did not cluster with known species or those described in the present study (Fig. 1).

Description of *Leptotrichia goodfellowii* sp. nov.

Leptotrichia goodfellowii (good.fel'low.i.i. N.L. gen. n. *goodfellowii* of Goodfellow, named in honour of Mike Goodfellow, for his contributions to microbial systematics).

After 2–6 days of anaerobic incubation at 37 °C, colonies on Columbia or BHI agar plates supplemented with 5% human blood, haemin and menadione are 0.8–2.0 mm in diameter, speckled, convex, irregular, pink in the periphery and greyish light brown in the rest of the colony. They have a glistening surface, are opaque, dry and β-haemolytic. Catalase positive and aesculin weakly positive. Oxidase and indole are not produced. Colonies grow best anaerobically and sparsely aerobically. Growth occurs at 37 °C but not at 25 or 42 °C; optimal temperature for growth is 37 °C. Gram-negative, non-spore-forming, non-motile rods. Cells

are arranged in pairs, some slightly curved, others in chains joined by flattened ends. Arginine dihydrolase, β-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, alkaline phosphatase, arginine arylamidase, leucine arylamidase and histidine arylamidase are produced. Mannose is fermented. Additional phenotypic properties are listed in Table 4. Isolated from human blood.

The type strain is LB 57^T (= CCUG 32286^T = CIP 107915^T).

Description of *Leptotrichia hofstadii* sp. nov.

Leptotrichia hofstadii (hof.stad'i.i. N.L. gen. n. *hofstadii* of Hofstad, named in honour of Tor Hofstad, for his contributions to *Leptotrichia* taxonomy).

After 2–6 days of anaerobic incubation at 37 °C, colonies on Columbia or BHI agar plates supplemented with 5% human blood, haemin and menadione are 0.5–1.8 mm in diameter. They have a glistening and granular surface, are opaque, dry and β-haemolytic. Older colonies can be up to 4.0–6.5 mm, circular, convex, entire (some are irregular and lobate) and greyish in colour with a dark central spot. Catalase positive and aesculin weakly positive. Oxidase and indole are not produced. Colonies grow best anaerobically and sparsely aerobically. Growth occurs at 37 °C but not at 25 or 42 °C; optimal temperature is 37 °C. Gram-negative, non-spore-forming, non-motile rods. Cells are arranged in pairs, some slightly curved, others in chains joined by flattened ends. β-Galactosidase-6-phosphate, α-glucosidase, β-glucosidase and alkaline phosphatase are produced. Mannose is fermented. Additional phenotypic properties are listed in Table 4. Isolated from the saliva of a healthy person.

The type strain is LB 23^T (= CCUG 47504^T = CIP 107917^T).

Description of *Leptotrichia shahii* sp. nov.

Leptotrichia shahii (shah'i.i. N.L. gen. n. *shahii* of Shah, named in honour of Haroun N. Shah, a Trinidad-born microbiologist, for his contributions to microbiology).

After 2–6 days of anaerobic incubation at 37 °C, colonies on Columbia or BHI agar plates supplemented with 5% human blood, haemin and menadione are 1.0–1.5 mm in diameter, very filamentous to rhizoid or convoluted, pale-speckled and greyish in colour, with a dark central spot in old colonies. These are opaque, semi-dry in consistency and non-haemolytic. Catalase positive and aesculin weakly positive. Oxidase and indole are not produced. Colonies grow best anaerobically and sparsely aerobically. Growth occurs at 25 and 37 °C but not at 42 °C; optimal temperature is 37 °C. Gram-negative, non-spore-forming, non-motile rods. Cells are arranged in pairs, some slightly curved, others in chains joined by flattened ends. α-Glucosidase and α-arabinosidase are produced. Additional phenotypic properties are listed in Table 4. Isolated from a patient with gingivitis.

Fig. 2. TEM (a, c, e and g) and SEM (b, d, f and h) of cells of novel *Leptotrichia* species from anaerobic cultures on Columbia blood agar grown at 37 °C for 48 h. (a), (c), (e) and (g) Cross-sectional views of cells of *L. goodfellowii* sp. nov. LB 57^T (a), *L. hofstadii* sp. nov. LB 23^T (c), *L. shahii* sp. nov. LB 37^T (e) and *L. wadei* sp. nov. LB 16^T (g). In (g), cells are visible in both a longitudinal (I) and a cross-sectional (II) view. G, Granule; IDL, intermediate dense layer; IM, inner cytoplasmic membrane; OM, outer membrane; S, septum; SLP, scale-like protrusion. Bars, 0.1 (a, e) and 0.25 (c, g) µm. (b), (d), (f) and (h) Cells from colonies of *L. goodfellowii* sp. nov. LB 57^T (b), *L. hofstadii* sp. nov. LB 23^T (d), *L. shahii* sp. nov. LB 37^T (f) and *L. wadei* sp. nov. LB 16^T (h) showing short (b, h) and long (d, f) rods. Bars, 2 (b) and 10 (d, f, h) µm.

The type strain is LB 37^T (=CCUG 47503^T=VPI N06A-34^T=CIP 107916^T).

Description of *Leptotrichia wadei* sp. nov.

Leptotrichia wadei (wade'i. N.L. gen. n. *wadei* of Wade, named in honour of William G. Wade, for his contributions to microbiology).

After 2–6 days of anaerobic incubation at 37 °C, colonies on Columbia or BHI agar plates supplemented with 5% human blood, haemin and menadione are 0.5–3.0 mm in diameter, convex, sparsely filamentous to irregular and greyish brown in colour, with a dark central spot in old colonies. The surface appearance is glistening and smooth with a rough edge. Colonies are opaque, dry in consistency and β -haemolytic. Aesculin and catalase are positive. Oxidase and indole are not produced. Growth occurs best anaerobically and sparsely aerobically at 37 °C but not at 25 or 42 °C. Gram-negative, non-spore-forming, non-motile rods. Cells are arranged in pairs, some slightly curved, others in chains joined by flattened ends. α -Glucosidase and β -glucosidase are produced. Additional phenotypic properties are listed in Table 4. Isolated from the saliva of a healthy person.

The type strain is LB 16^T (=CCUG 47505^T=CIP 107918^T).

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