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

Emenike R. K. Eribe,1 Bruce J. Paster,2,3 Dominique A. Caugant,1,4 Floyd E. Dewhirst,2,3 Verlyn K. Stromberg,5 George H. Lacy5 and Ingar Olsen1

Correspondence
Emenike R. K. Eribe
emenike@odont.uio.no

1Institute of Oral Biology, Dental Faculty, University of Oslo, POB 1052, Blindern, N-0316 Oslo, Norway
2Department of Molecular Genetics, The Forsyth Institute, 140 The Fenway, Boston, MA 02115, USA
3Department of Oral and Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, USA
4Division for Infectious Disease Control, Norwegian Institute of Public Health, POB 4404, Nydalen, N-0403 Oslo, Norway
5Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0330, USA

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 57T = CCUG 32286T = CIP 107915T), *Leptotrichia hofstadii* sp. nov. (type strain LB 23T = CCUG 47504T = CIP 107917T), *Leptotrichia shahii* sp. nov. (type strain LB 37T = CCUG 47503T = CIP 107916T) and *Leptotrichia wadei* sp. nov. (type strain LB 16T = CCUG 47505T = CIP 107918T). 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% DNA–DNA relatedness, *L. shahii* showed 24%–34%–1% relatedness, *L. hofstadii* showed 27%–36%–3% relatedness and *L. wadei* showed 24%–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 Published online ahead of print on 21 November 2003 as DOI 10.1099/ijs.0.02819-0.
The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *L. hofstadii* LB 23T, *L. shahii* LB 37T, *L. wadei* LB 16T and *L. goodfellowii* LB 57T 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-sporeulating 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 perirectal 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; Messiain 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 rRNA 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 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 subclassed anaerobically on Columbia blood agar plates at 37°C for 2–5 days. After culturing, bacterial cells were harvested by centrifugation (11000 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 Taqstart 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 4–5 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 Sequenex 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 Dewhurst’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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference no.</th>
<th>Source and other designation</th>
<th>Site of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB 1</td>
<td>1-2</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Gingival pocket, periodontitis</td>
</tr>
<tr>
<td>LB 2</td>
<td>1-4</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Gingival pocket, periodontitis</td>
</tr>
<tr>
<td>LB 3</td>
<td>8-1</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Gingival pocket, periodontitis</td>
</tr>
<tr>
<td>LB 4</td>
<td>11-1</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Gingival pocket, periodontitis</td>
</tr>
<tr>
<td>LB 5</td>
<td>11-2</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Gingival pocket, periodontitis</td>
</tr>
<tr>
<td>LB 6</td>
<td>82A-026</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 7</td>
<td>89A-069</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 8</td>
<td>90-0274</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 9</td>
<td>90-0309</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 10</td>
<td>91A064</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 11</td>
<td>94-0155</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 12</td>
<td>94-0200</td>
<td>K. Bernard, Ottawa, Ontario, Canada</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 13</td>
<td>ATCC 14201T</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Supragingival calculus</td>
</tr>
<tr>
<td>LB 14</td>
<td>ES 23-91</td>
<td>R. Hammann, Heidelberg, Germany</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 15</td>
<td>L 11</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 16</td>
<td>L 41</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 17</td>
<td>L 42</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 18</td>
<td>L 43</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 19</td>
<td>L 44</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 20</td>
<td>L 45</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 21</td>
<td>L 46</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 22</td>
<td>L 47</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 23</td>
<td>L 48</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 24</td>
<td>L 49</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 25</td>
<td>L 50</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 26</td>
<td>L 51</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 27</td>
<td>L 90</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 28</td>
<td>L 92</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 29</td>
<td>L 101</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 30</td>
<td>L 102</td>
<td>Gade Institute, Bergen, Norway</td>
<td>Healthy person, saliva</td>
</tr>
<tr>
<td>LB 31</td>
<td>RYC 29853</td>
<td>M. Reig, Madrid, Spain</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 32</td>
<td>RYC 30220</td>
<td>M. Reig, Madrid, Spain</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 33</td>
<td>ATCC 19616</td>
<td>Gade Institute, Bergen, Norway</td>
<td>NK</td>
</tr>
<tr>
<td>LB 37</td>
<td>N06A-34</td>
<td>VPI, Blacksburg, VA, USA</td>
<td>Gingivitis</td>
</tr>
<tr>
<td>LB 47</td>
<td>W16A-16</td>
<td>VPI, Blacksburg, VA, USA</td>
<td>Juvenile periodontitis</td>
</tr>
<tr>
<td>LB 48</td>
<td>CCUG 19067B</td>
<td>Danderyd PHL, Danderyd, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 49</td>
<td>CCUG 19169</td>
<td>M. Wickström, Göteborg, Sweden</td>
<td>Subgingival pocket</td>
</tr>
<tr>
<td>LB 50</td>
<td>CCUG 19427</td>
<td>Lund PHL, Lund, Sweden</td>
<td>NK</td>
</tr>
<tr>
<td>LB 51</td>
<td>CCUG 21070</td>
<td>Uddevalla PHL, Uddevalla, Sweden</td>
<td>Blood, leukaemia</td>
</tr>
<tr>
<td>LB 52</td>
<td>CCUG 23775</td>
<td>PHL, Göteborg, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 53</td>
<td>CCUG 29716</td>
<td>Ha, Uppsala, Sweden</td>
<td>NK</td>
</tr>
<tr>
<td>LB 54</td>
<td>CCUG 30637</td>
<td>Gävle, Sweden</td>
<td>NK</td>
</tr>
<tr>
<td>LB 55</td>
<td>CCUG 32116</td>
<td>Gävle PHL, Gävle, Sweden</td>
<td>Synovia, septic arthritis</td>
</tr>
<tr>
<td>LB 56</td>
<td>CCUG 32173</td>
<td>PHL, Göteborg, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 57</td>
<td>CCUG 32286</td>
<td>Heidelberg, Germany</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 58</td>
<td>CCUG 33471</td>
<td>PHL, Göteborg, Sweden</td>
<td>Human</td>
</tr>
<tr>
<td>LB 59</td>
<td>CCUG 34278</td>
<td>PHL, Göteborg, Sweden</td>
<td>Human</td>
</tr>
<tr>
<td>LB 61</td>
<td>CCUG 34631</td>
<td>PHL, Sundsvall, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 62</td>
<td>CCUG 35982</td>
<td>Karlstad PHL, Karlstad, Sweden</td>
<td>Blood, acute leukaemia</td>
</tr>
<tr>
<td>LB 63</td>
<td>CCUG 36771</td>
<td>Uppsala, Sweden</td>
<td>Blood</td>
</tr>
</tbody>
</table>
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 [$\alpha$-33P]dCTP (Perkin Elmer Life Sciences). The probe and target DNAs were reassociated at $66 \pm 0.5^\circ C$ 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.

**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 57T), Leptotrichia hofstadii sp. nov. (LB 23T), Leptotrichia shahii sp. nov. (LB 37T) and Leptotrichia wadei sp. nov. (LB 16T). 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 16T, from the saliva of a healthy man, clustered with a previously described strain, A39FD, that was isolated from subgingival plaque (Fig. 1). LB 16T had 94 % sequence similarity to L. buccalis.

The type strains of L. hofstadii (LB 23T), L. shahii (LB 37T) and L. goodfellowii (LB 57T) each formed individual clusters that respectively showed 97, 96 and 92 % sequence similarity to L. buccalis. L. hofstadii LB 23T, isolated from the saliva of a healthy man, clustered with a previously described strain (FAC5) that was isolated from dental plaque. L. shahii LB 37T, from dental plaque of a gingivitis patient, clustered with DE081, a previously described phylotype from dental plaque. L. goodfellowii LB 57T 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>
<thead>
<tr>
<th>Strain</th>
<th>Reference no.</th>
<th>Source and other designation</th>
<th>Site of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB 64</td>
<td>CCUG 36868</td>
<td>Tromsø, Norway</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 65</td>
<td>CCUG 36980</td>
<td>Skövde PHL, Skövde, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 66</td>
<td>CCUG 38157</td>
<td>PHLS, Halmstad, Sweden</td>
<td>Blood, leukaemia</td>
</tr>
<tr>
<td>LB 67</td>
<td>CCUG 38343</td>
<td>Lindköping, Sweden</td>
<td>Sputum</td>
</tr>
<tr>
<td>LPB 68</td>
<td>CCUG 32006</td>
<td>Sundsvall, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LPB 69</td>
<td>CCUG 34137</td>
<td>PHL, Danderyd, Sweden</td>
<td>Blood</td>
</tr>
<tr>
<td>LB 70</td>
<td>ATCC 23471</td>
<td>G. Frostell strain 18, Sweden</td>
<td>Oral cavity</td>
</tr>
<tr>
<td>LB 71</td>
<td>ATCC 23472</td>
<td>G. Frostell strain 19, Sweden</td>
<td>Oral cavity</td>
</tr>
<tr>
<td>LB 76</td>
<td>CCUG 41699</td>
<td>PHLS, Halmstad, Sweden</td>
<td>Blood, pneumonia</td>
</tr>
<tr>
<td>LB 77</td>
<td>CCUG 42664</td>
<td>PHL, Karlstad, Sweden</td>
<td>Blood, leukaemia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Function</th>
<th>Position (bp)*</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9F</td>
<td>Forward</td>
<td>PCR</td>
<td>9–27</td>
<td>GAGTTTGATYCTGGCTCAG</td>
</tr>
<tr>
<td>338F</td>
<td>Forward</td>
<td>Sequencing</td>
<td>339–358</td>
<td>CTCCTACGGGAGGGACAGT</td>
</tr>
<tr>
<td>531R</td>
<td>Reverse</td>
<td>Sequencing</td>
<td>509–527</td>
<td>TACCGCGGCTGCTGGCAC</td>
</tr>
<tr>
<td>789F</td>
<td>Forward</td>
<td>Sequencing</td>
<td>789–806</td>
<td>TAGATACCCYGGTAGTCC</td>
</tr>
<tr>
<td>926R</td>
<td>Reverse</td>
<td>Sequencing</td>
<td>896–915</td>
<td>CCGTCWATCMITTTGAGT</td>
</tr>
<tr>
<td>1099F</td>
<td>Forward</td>
<td>Sequencing</td>
<td>1099–1113</td>
<td>GCAACGAGCCGCACC</td>
</tr>
<tr>
<td>1193R</td>
<td>Reverse</td>
<td>Sequencing</td>
<td>1175–1194</td>
<td>ACGTATCCCAACCTTCTC</td>
</tr>
<tr>
<td>1541R</td>
<td>Reverse</td>
<td>PCR</td>
<td>1526–1541</td>
<td>GAAGGAGGTGWTCCACCCGCA</td>
</tr>
</tbody>
</table>

*Numbered relative to Escherichia coli (Woese et al., 1983).
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 13T, LB 16, LB 23, LB 37T, LB 57T and *L. trevisanii* LTT) 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* LTT. 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 23T, LB 37T and LB 57T. 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 (≥70%) groupings are boxed. E. amylovora was included as an outgroup.

<table>
<thead>
<tr>
<th>Source of labelled DNA</th>
<th>DNA–DNA relatedness with unlabelled DNA from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. L. buccalis LB13T</td>
<td>100</td>
</tr>
<tr>
<td>2. L. trevisanii LT4T</td>
<td>42</td>
</tr>
<tr>
<td>3. Strain LB 6</td>
<td>40</td>
</tr>
<tr>
<td>4. Strain LB 11</td>
<td>36</td>
</tr>
<tr>
<td>5. L. wadei LB 16T</td>
<td>24</td>
</tr>
<tr>
<td>6. L. hofstadii LB 23T</td>
<td>36</td>
</tr>
<tr>
<td>7. L. shahii LB 37T</td>
<td>24</td>
</tr>
<tr>
<td>8. L. goodfellowii LB 57T</td>
<td>5</td>
</tr>
<tr>
<td>9. Erwinia amylovora EA 110</td>
<td>-4</td>
</tr>
</tbody>
</table>

Table 4. Salient characteristics of Leptotrichia species

Strains: 1. L. buccalis LB 13T; 2. L. goodfellowii LB 57T; 3. L. hofstadii LB 23T; 4. L. shahii LB 37T; 5. L. wadei LB 16T. 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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains</td>
<td>26</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Number of clones</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>42°C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Haemolysis of human full blood</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ID 32 A results:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Galactosidase-6-phosphate</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>α-Arabinosidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannose fermentation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose fermentation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arginine arylamidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine arylamidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Histidine arylamidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Profile</td>
<td>4616400100 2111412001 0612400000 0420000000 0410000000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated straight-chain fatty acids:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>10</td>
<td>14</td>
<td>6</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>16:0</td>
<td>39</td>
<td>41</td>
<td>48</td>
<td>32</td>
<td>45</td>
</tr>
<tr>
<td>Unsaturated straight-chain fatty acids:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1c11/19/6 or unknown with ECL of 17-834</td>
<td>42</td>
<td>28</td>
<td>27</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>Hydroxy fatty acids:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-OH-14:0 or 15:0 DMA</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>
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 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 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 human blood.

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

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 57T (a), *L. hofstadii* sp. nov. LB 23T (c), *L. shahii* sp. nov. LB 37T (e) and *L. wadei* sp. nov. LB 16T (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 57T (b), *L. hofstadii* sp. nov. LB 23T (d), *L. shahii* sp. nov. LB 37T (f) and *L. wadei* sp. nov. LB 16T (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\textsuperscript{T} (=CCUG 47503\textsuperscript{T} = VPI N06A-34\textsuperscript{T} = CIP 107916\textsuperscript{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 BH\textsubscript{I} 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\textsuperscript{T} (=CCUG 47505\textsuperscript{T} = CIP 107918\textsuperscript{T}).

**ACKNOWLEDGEMENTS**

We are grateful to W. E. C. Moore (deceased) and L. V. H. Moore, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, to E. Falsen, CCUG Göteborg, Sweden, and to T. Hofstad, The Gade Institute, Bergen, Norway, for their donations of strains. Thanks are also due to T. Alvestad and A.-M. Klem, Division of Infectious Control, Norwegian Institute of Public Health, Oslo, and to S. K. Boches and J. L. Galvin, Forsyth Institute, Boston, MA, USA, for their assistance with microbiology.

**REFERENCES**


