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Leptotrichia hongkongensis sp. nov., a novel *Leptotrichia* species with the oral cavity as its natural reservoir*

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Abstract: A straight, non-sporulating, Gram-variable bacillus (HKU24^T) was recovered from the blood culture of a patient with metastatic breast carcinoma. After repeated subculturing in BACTEC Plus Anaerobic/F blood culture broth, HKU24^T grew on brucella agar as non-hemolytic, pinpoint colonies after 96 h of incubation at 37 °C in an anaerobic environment and aerobic environment with 5% CO_2. Growth was enhanced with a streak of *Staphylococcus aureus*. HKU24^T was non-motile and catalase-negative, but positive for alkaline phosphatase, β-glucosidase, and α-glucosidase. It hydrolyzed phenylphosphonate and reduced resazurin. 16S rRNA, *groEL*, *gyrB*, *recA*, and *rpoB* sequencing showed that HKU24^T occupies a distinct phylogenetic position among the *Leptotrichia* species, being most closely related to *Leptotrichia trevisanii*. Using HKU24^T *groEL, gyrB, recA*, and *rpoB* gene-specific primers, fragments of these genes were amplified from one of 20 oral specimens. Based on phenotypic and genotypic characteristics, we propose a new species, *Leptotrichia hongkongensis* sp. nov., to describe this bacterium.

Key words: *Leptotrichia hongkongensis*, Bacterium, Blood culture, Anaerobic bacteria, Novel species
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1 Introduction

Leptotrichia species are Gram-variable, non-sporulating, large, fusiform, non-motile bacilli. Since the first description in 1879 (Trevisan, 1879), *Leptotrichia buccalis* has been the only species in the genus *Leptotrichia* until 2001 (Tee *et al*., 2001). *L. buccalis* is part of the human normal oral flora and has been implicated as a cause of dental infections and bacteremia after tooth extractions (Crawford *et al*., 1974; Munson *et al*., 2004; Sutter, 1984). However, severe systemic infections associated with *L. buccalis* have been uncommon. Most of the serious cases occurred as bacteremia in patients with malignancies, with a significant proportion having neutropenia (Eribe and Olsen, 2008; Morgenstein *et al*., 1980; Reig *et al*., 1985; Schwartz *et al*., 1995; Ulstrup and Hartzen, 2006; Weinberger *et al*., 1991). Case reports of infective endocarditis associated with *L. buccalis* in patients with prosthetic heart valves or congenital heart defects have also been described (Caram *et al*.,

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2008; Duperval *et al*., 1984; Eribe and Olsen, 2008; Hammann *et al*., 1993). Since 2001, five additional species of *Leptotrichia*, including *L. trevisanii*, *L. goodfellowii*, *L. hofstadii*, *L. shahii*, and *L. wadei*, have been described (Eribe *et a*l., 2004; Tee *et al*., 2001). All these five novel species have been isolated from human specimens, some from blood cultures and others from the oral cavity.

Recently, we isolated a bacterial strain, named HKU24^T, from the blood culture of a patient with metastatic carcinoma of the breast. The strain exhibited distinct phenotypic characteristics that did not fit into patterns of any known bacterial species. Based on these observations, we hypothesized that HKU24^T may represent a novel bacterial species. To test the hypothesis, we sequenced the 16S rRNA gene and four additional housekeeping genes (frequently used for bacterial identification and phylogenetic studies) of HKU24^T and four closely-related *Leptotrichia* species identified by 16S rRNA gene sequencing. On the basis of both the phenotypic and genotypic characteristics, we proposed a new species, *Leptotrichia hongkongensis* sp. nov., to describe this bacterium.

2 Materials and methods

2.1 Patient and strains

Strain HKU24^T was isolated from the blood culture of a patient with disseminated carcinoma of the breast. *L. buccalis* (HKU27) was isolated from the blood culture of a patient in Hong Kong (Woo *et al*., 2003b). *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), and *L. trevisanii* (CCUG 49525) were obtained from the Culture Collection, University of Gőteborg (CCUG), Sweden.

2.2 Phenotypic characterization

Clinical specimens were collected and handled according to standard protocols (Murray *et al*., 2007). Blood cultures were performed with the BACTEC 9240 system with Plus Aerobic/F and Plus Anaerobic/F bottles (Becton Dickinson Microbiology Systems, Sparks, Md., USA). All suspect colonies were identified by standard conventional biochemical methods (Murray *et al*., 2007). All tests were performed in triplicate with freshly prepared media on separate occasions. In addition, the Vitek system (ANI and NH; bioMerieux Vitek, USA) was used for the identification of HKU24^T. In vitro susceptibilities to penicillin, metronidazole, vancomycin, amoxicillin-clavulanate, and imipenem were determined using the E-test method.

2.3 Scanning electron microscopy (SEM)

Bacterial cells were washed twice using Milli-Q water. A suspension of the bacterium was settled onto a polycarbonate membrane (Nucleopore, USA) with a pore size of 5 μm for 5 min. The membrane was fixed in 2.5% (w/v) glutaraldehyde for 1 h and washed once in 0.1 mol/L sodium cacodylate buffer. Fixed material was dehydrated through a graded ethanol series from 30% to 90% in 20% steps, followed by two changes of absolute ethanol. Each of the stepwise changes was for 15 min. Dehydrated material in absolute ethanol was critical point-dried in a BAL-TEC CPD O30 Critical Point Drier using carbon dioxide as the drying agent. Critical point-dried material was mounted on to an aluminum stub and coated with palladium in BAL-TEC SCD 005 SEM coating system. Coated material was examined in Leica Cambridge Stereoscan 440 SEM operating at 12 kV and the specimen stage was tilted at zero degree (Woo *et al*., 2002c).

2.4 DNA extraction

Bacterial DNA extraction was performed according to our previous publication (Woo *et al*., 2009). Briefly, 80 μl of NaOH (0.05 mol/L) was added to 20 μl of bacterial cells suspended in distilled water and the mixture was incubated at 60 °C for 45 min, followed by addition of 6 μl of Tris-HCl (pH 7.0), achieving a final pH of 8.0. The resultant mixture was diluted 100× and 5 μl of the diluted extract was used for polymerase chain reaction (PCR).

2.5 Sequencing of 16S rRNA, *groEL*, *gyrB*, *recA*, and *rpoB* genes

PCR amplification and DNA sequencing of the 16S rRNA, *groEL*, *gyrB*, *recA*, and *rpoB* genes of HKU24^T, *L. buccalis* (HKU27), *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), and *L. trevisanii* (CCUG 49525) were performed according to our previous publications on other anaerobic bacteria (Lau *et al*., 2004; Woo *et al*., 2002a; 2002b; 2003a; 2004a; 2007). Briefly, DNase I-treated distilled water and PCR master mix, which contains deoxynucleoside

triphosphates (dNTPs), PCR buffer, and *Taq* polymerase, were used in all PCR reactions by adding 1.0 U of DNase I (Invitrogen, USA) to 40 μl of distilled water or PCR master mix, and then incubating the mixture at 25 °C for 15 min and subsequently at 95 °C for 10 min to inactivate the DNase I. The bacterial DNA extract and control were amplified with 0.5 μmol/L primers (Table 1). The PCR mixture (50 μl) contained bacterial DNA, PCR buffer (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, and 0.01% (w/v) gelatin), 200 μmol/L of each dNTPs, and 1.0 U *Taq* polymerase (Applied Biosystems, Foster City, CA, USA). The mixtures were amplified in 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min in an automated thermal cycler (Applied Biosystems). DNase I-treated distilled water was used as the negative control. A total of 10 μl of each amplified product was electrophoresed in 1.5% (w/v) agarose gel, with a molecular size marker [PhiX174 DNA/*Bsu*RI (*Hae*III) Marker, Fermentas, Germany] in parallel. Electrophoresis in Tris-borate-ethylene diamine tetraacetic acid (EDTA) buffer was performed at 100 V for 1.5 h. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min, rinsed, and photographed under ultraviolet light illumination.

The PCR product was gel-purified using the QIAquick PCR Purification Kit (QIAgen, Hilden, Germany). Both strands of the PCR products were sequenced twice with an ABI Prism 3700 DNA Analyzer (Applied Biosystems), using the PCR primers. The sequences of the PCR products were compared with sequences of closely related species in GenBank by multiple sequence alignment using ClustalX 1.83 (Thompson *et al*., 1997).

2.6 Detection of *Leptotrichia hongkongensis* from oral specimens

Sterile polyester swabs were used to gently rub the oral mucosa and tooth surfaces of 20 healthy volunteers who were not on antibiotics in the past four weeks. Samples were suspended in 3 ml of saline followed by culturing in BACTEC Plus Anaerobic/F blood culture broth containing 40 μg/ml of metronidazole and 8 μg/ml of levofloxacin at 37 °C for 72 h. DNA was extracted from 1 ml of cultured bacterial cells using the method described above and the DNA extract further purified using QIAquick PCR Purification Kit (QIAgen).

Using *L. hongkongensis* gene specific primers (Table 1), a 135-bp fragment of the *groEL* gene, 269-bp fragment of the *gyrB* gene, 307-bp fragment of the *recA* gene, and 433-bp fragment of the *rpoB* gene of *L. hongkongensis* were amplified from the DNA extracts of the bacterial cells recovered from the oral specimens of the 20 healthy volunteers in four separate PCR reactions, using *L. hongkongensis* type strain HKU24^T as the positive control and distilled water as the negative control. The PCR mixture (50 μl) contained bacterial DNA, PCR buffer (10 mmol/L

Table 1 Primers used in this study

Gene locus	Primers		Amplicon size (bp)
	Forward	Reverse	
16S rRNA	LPW8385 5′-GAACGCTGACAGAATGCTTA-3′	LPW8387 5′-CCAATCACTATCCACACCTTA-3′	1425
groEL	LPW8389 5′-GTTGTGGAAGGNATGCARTTYGA-3′	LPW8441 5′-CAGCTCCAACTTTTATTACAGCT-3′	555
gyrB	LPW10271 5′-GGAAMWGAYRTAAGAGAAGG-3′	LPW8399 5′-TTCATTTCTCCTAGNCCYTTRTA-3′	796
recA	LPW8402 5′-GGTGCCGTTATGAAAYTNGGNGA-3′	LPW10124 5′-GAACCAGGCTCCAGCTTT-3′	813
rpoB	LPW8697 5′-AAATGGCACTTGAGCTGT-3′	LPW8698 5′-CAATTCCAACAGTAATTCCA-3′	768
groEL[a]	LPW10130 5′-ATAATCGCTGAAGATGTG-3′	LPW10132 5′-TAACTTCTCCACCTGTTAAT-3′	158
gyrB[a]	LPW11369 5′-AAGCAACTTGAAATTCTATCTA-3′	LPW11372 5′-ATATTGCCTGAAATCTTCTG-3′	271
recA[a]	LPW11373 5′-CAGAAGGCTGGAGGAACG-3′	LPW11376 5′-AGATTTTGAGATACTTCCTGTT-3′	308
rpoB[a]	LPW11377 5′-ACCATCGAGTGGTAGACCA-3′	LPW11380 5′-CTAATTCACCTTTACCAAAA-3′	285

[a] Gene specific primers for the detection of *L. hongkongensis* from an oral specimen

Tris-HCl pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, and 0.01% (w/v) gelatin), 200 μmol/L of each dNTPs, and 1.0 U *Taq* polymerase (Applied Biosystems). The mixtures were amplified in 60 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min in an automated thermal cycler (Applied Biosystems). Agarose gel electrophoresis, purification of PCR products, and DNA sequencing were performed as described above.

2.7 Phylogenetic characterization

Phylogenetic tree construction was performed using the neighbor joining method with ClustalX 1.83 (Thompson *et al*., 1997). One thousand three hundred and eleven nucleotide positions of the 16S rRNA gene and 519, 692, 716, and 646 nucleotide positions of the *groEL*, *gyrB*, *recA*, and *rpoB* genes of HKU24^T, respectively, were included in the analysis. A total of 158, 271, 308, and 244 nucleotide positions of the *groEL*, *gyrB*, *recA*, and *rpoB* genes, respectively, were included in the analysis of the sequences amplified from the oral specimen.

2.8 Nucleotide sequence accession numbers

The 16S rRNA, *groEL*, *gyrB*, *recA*, and *rpoB* gene sequences of *L. hongkongensis* (HKU24^T), *L. buccalis* (HKU27), *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), and *L. trevisanii* (CCUG 49525) have been lodged within the GenBank sequence database under accession numbers EU919515 and GU086169 to GU086196.

3 Results

3.1 Patient

A 66-year-old Chinese woman was admitted because of fever for three days. The patient had carcinoma of the left breast five years ago, having been treated with modified radical mastectomy, chemotherapy, and hormonal therapy. After defaulting further hormonal therapy for two years, the patient developed metastatic lesions in the lungs, pleura, lymph nodes, and multiple ribs one year ago, and was treated with multiple courses of chemotherapy. She also had histories of bilateral retinal detachment and cataract 20 years ago and colonic polyp. Four days before the scheduled 10th course of chemotherapy, she developed fever. On admission, her body temperature was 38.9 °C. Examination did not reveal any localizing signs. Total white cell count was 7.2×10^9 L^{-1} (neutrophil count of 5.5×10^9 L^{-1}, lymphocyte count 0.8×10^9 L^{-1}). Liver and renal function tests were normal. Blood culture was performed and empirical intravenous amoxicillin-clavulanate was commenced.

On Day 2 post-incubation, the anaerobic blood culture bottle turned positive with a non-sporulating Gram-variable bacillus (strain HKU24^T) (Fig. 1a). The fever gradually subsided after three days of amoxicillin-clavulanate and antibiotics were continued for a total of 14 d. There was no relapse of the bacteremia up to the time of writing, one year after discharge.

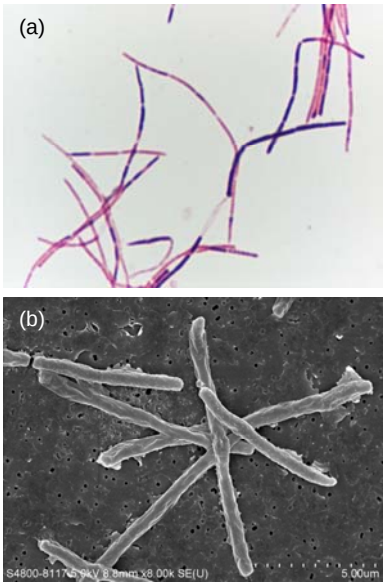

Fig. 1 Gram smear and scanning electron micrograph of HKU24^T. (a) Gram smear showing a straight, non-sporulating, Gram-variable bacillus; (b) Scanning electron micrograph showing that the bacterium is aflagellate and straight

Cells vary in length from 5.86 to 11.94 μm and diameter from 0.50 to 0.74 μm (mean=8.92 μm×0.62 μm, *n*=20)

3.2 Phenotypic characteristics

Strain HKU24^T is a straight, non-sporulating, Gram-variable bacillus (Fig. 1a). On primary subculture, it does not grow on sheep blood agar, chocolate agar, or brucella agar. After repeated subculturing in BACTEC Plus Anaerobic/F blood culture broth, it grows on brucella agar as non-hemolytic, pinpoint colonies after 96 h of incubation at 37 °C in anaerobic environment, as well as the aerobic environment with 5% CO_2. Growth is enhanced with a

streak of *Staphylococcus aureus*. It is non-motile. It dose not produce catalase. The Vitek system (ANI and NH) showed that it was positive for alkaline phosphatase, β-glucosidase, and α-glucosidase. It hydrolyzed phenylphosphonate and reduced resazurin (Table 2). The minimal inhibitory concentrations of penicillin, metronidazole, vancomycin, amoxicillin-clavulanate, levofloxacin, and imipenem for the isolate were 0.006, 8, 0.5, 0.016, >32, and 0.012 μg/ml, respectively.

3.3 Scanning electron microscopy

A scanning electron micrograph of HKU24^T is shown in Fig. 1b. Bacterial cells were aflagellate straight bacilli.

Table 2 Biochemical profiles of the blood culture isolate HKU24^T by Vitek ANI and NH systems

Biochemical reactions/ enzymes/substrates	Vitek ANI	Vitek NH
Arginine dehydrogenase	−	
Ornithine decarboxylase		−
Alkaline phosphatase	+	
Phosphate choline	−	−
Urease	−	−
Penicillinase		−
Phenylphosphonate		+
Reduction of triphenyltetrazolium	−	−
Reduction of resazurin		+
Oxidation/fermentation of:		
Arabinose	−	
Glucose	−	−
Raffinose	−	
Trehalose	−	
Xylose	−	
α-arabinosidase	−	
α-fucosidase	−	
β-fucosidase	−	
α-galactosidase	−	
β-galactosidase	−	−
α-glucosidase	+	
β-glucosidase	+	
β-glucuronidase	−	
α-mannosidase	−	
β-lactosidase	−	
β-xylosidase	−	
N-acetyl-glucosaminidase	−	
Alanine arylamidase	−	−
Benzoyl-arginine arylamidase	−	
Gamma-glutamyl-arylamidase	−	−
Leucine arylamidase	−	
Lysine arylamidase	−	−
Proline arylamidase	−	−
Glycine arylamidase		−

3.4 Molecular and phylogenetic characterization by 16S rRNA gene sequencing

Sequencing of the 16S rRNA gene of HKU24^T showed that there were two (0.15%) base differences between the 16S rRNA gene sequence of HKU24^T and that of an unnamed bacterium isolated in 1999 (AF189244), five (0.38%) between the 16S rRNA gene sequence of HKU24^T and that of *Leptotrichia* species oral clone DA 069 (AF287811), six (0.45%) between the 16S rRNA gene sequence of HKU24^T and that of an uncultured *Leptotrichia* species oral clone 502H05 (AM420200), 34 (2.59%) between the 16S rRNA gene sequence of HKU24^T and that of *L. trevisanii* (AY029801), 46 (3.51%) between the 16S rRNA gene sequence of HKU24^T and that of *L. shahii* (NR_025648), 59 (4.50%) between the 16S rRNA gene sequence of HKU24^T and that of *L. wadei* (AY029802), 70 (5.33%) between the 16S rRNA gene sequence of HKU24^T and that of *L. hofstadii* (AY029803), 83 (6.33%) between the 16S rRNA gene sequence of HKU24^T and that of *L. buccalis* (L37788.1), and 141 (10.76%) base differences between the 16S rRNA gene sequence of HKU24^T and that of *L. goodfellowii* (AY029807) (Fig. 2).

3.5 Phylogenetic characterization by *groEL*, *gyrB*, *recA*, and *rpoB* gene sequencing

PCR of the partial *groEL* genes of HKU24^T, *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), *L. trevisanii* (CCUG 49525), and *L. buccalis* (HKU27) showed bands at about 550 bp. There were 22 (4.24%) base differences between the *groEL* gene of HKU24^T and that of *L. buccalis* (DSM1135), 26 (5.01%) between the *groEL* gene of HKU24^T and that of *L. buccalis* (HKU27), 35 (6.74%) between the *groEL* gene of HKU24^T and that of *L. trevisanii* (CCUG 49525), 56 (10.79%) between the *groEL* gene of HKU24^T and that of *L. wadei* (CCUG 47505), and 68 (13.10%) between the *groEL* gene of HKU24^T and that of *L. shahii* (CCUG 47503) (Fig. 3a).

PCR of the partial *gyrB* genes of HKU24^T, *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), *L. trevisanii* (CCUG 49525), and *L. buccalis* (HKU27) showed bands at about 800 bp. There were 40 (5.78%) base differences between the *gyrB* gene of HKU24^T and that of *L. trevisanii* (CCUG 49525), 47 (6.79%) between the *gyrB* gene of HKU24^T and that of *L. buccalis* (DSM1135), 62 (8.96%) between the *gyrB*

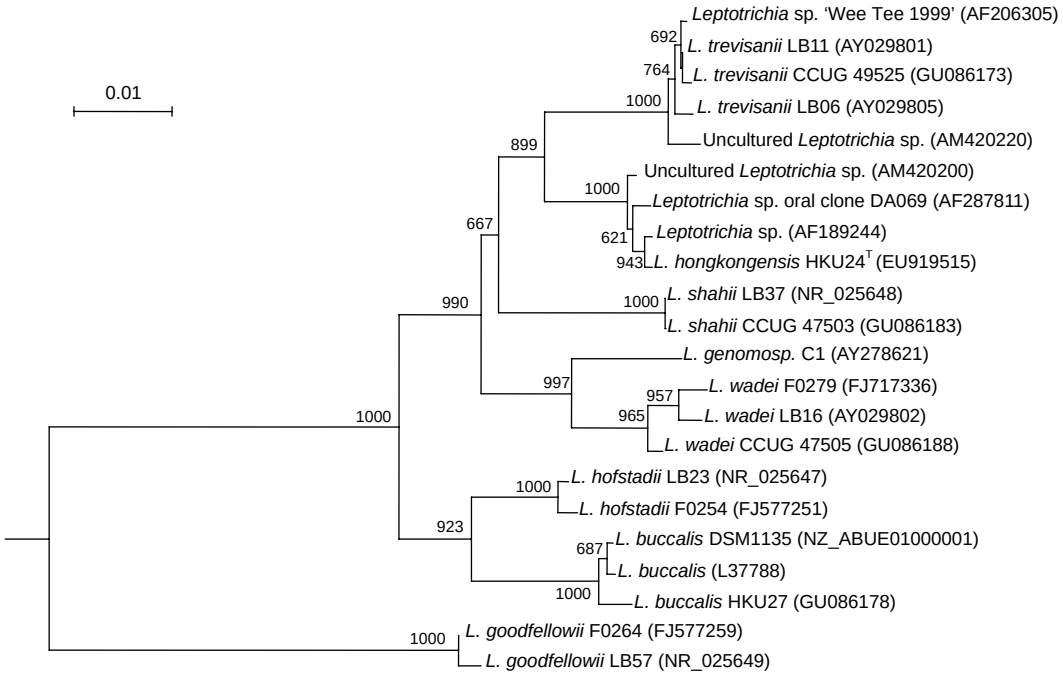

Fig. 2 Phylogenetic relationship of 16S rRNA between the *L. hongkongensis* HKU24^T and other *Leptotrichia* species. The tree was inferred from 16S rRNA data by the neighbor-joining method and rooted using the 16S rRNA gene sequence of *Fusobacterium nucleatum* (FJ471645). Bootstrap values were calculated from 1000 trees. The scale bar indicates the estimated number of substitutions per 100 bases. Names and accession numbers are given as cited in the GenBank database

gene of HKU24^T and that of *L. buccalis* (HKU27), 74 (10.69%) between the *gyrB* gene of HKU24^T and that of *L. wadei* (CCUG 47505), and 79 (11.42%) between the *gyrB* gene of HKU24^T and that of *L. shahii* (CCUG 47503) (Fig. 3b).

PCR of the partial *recA* genes of HKU24^T, *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), *L. trevisanii* (CCUG 49525), and *L. buccalis* (HKU27) showed bands at about 800 bp. There were 56 (7.82%) base differences between the *recA* gene of HKU24^T and that of *L. trevisanii* (CCUG 49525), 67 (9.36%) between the *recA* gene of HKU24^T and that of *L. shahii* (CCUG 47503), 75 (10.47%) between the *recA* gene of HKU24^T and that of *L. wadei* (CCUG 47505), and 80 (11.17%) between the *recA* gene of HKU24^T and those of *L. buccalis* (HKU27) and *L. buccalis* (DSM1135) (Fig. 3c).

PCR of the partial *rpoB* genes of HKU24^T, *L. shahii* (CCUG 47503), *L. wadei* (CCUG 47505), *L. trevisanii* (CCUG 49525), and *L. buccalis* (HKU27) showed bands at about 750 bp. There were 59 (9.13%) base differences between the *rpoB* gene of HKU24^T and that of *L. buccalis* (DSM1135), 60 (9.29%) between the *rpoB* gene of HKU24^T and that of *L. buccalis* (HKU27), 75 (11.61%) between the *rpoB* gene of HKU24^T and that of *L. trevisanii* (CCUG 49525), 118 (18.27%) between the *rpoB* gene of HKU24^T and that of *L. wadei* (CCUG 47505), and 130 (20.12%) between the *rpoB* gene of HKU24^T and that of *L. shahii* (CCUG 47503) (Fig. 3d).

3.6 Detection of *Leptotrichia hongkongensis* from oral specimens

Using *L. hongkongensis* gene specific primers, the 158-bp, 271-bp, 308-bp, and 285-bp fragments of the *groEL*, *gyrB*, *recA*, and *rpoB* genes, respectively, could be amplified from the DNA extracted from bacterial cells recovered from one of the 20 oral specimens from healthy volunteers. Sequencing of the purified PCR products showed that there were 0, 7, 1, and 18 base differences between the 158-bp, 271-bp, 308-bp, and 244-bp fragments of the *groEL*, *gyrB*, *recA*, and *rpoB* genes, respectively, amplified from the DNA extracted from bacterial cells recovered from the oral specimen and the corresponding region of *L. hongkongensis* strain HKU24^T (Fig. 4).

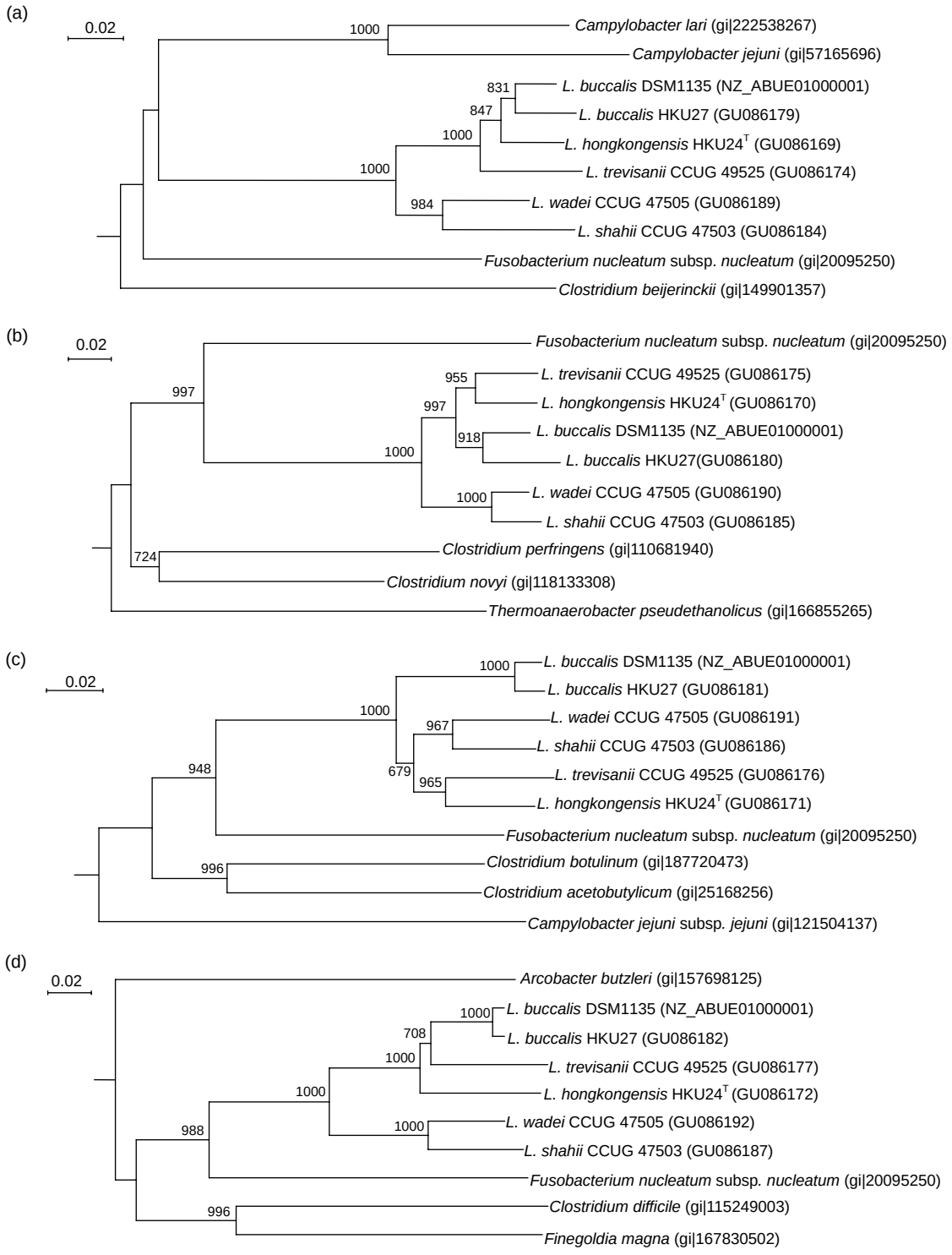

Fig. 3 Phylogenetic relationships of *groEL* (a), *gyrB* (b), *recA* (c), and *rpoB* (d) genes between the *L. hongkongensis* HKU24^T and other *Leptotrichia* species. The trees were constructed by the neighbor-joining method and bootstrap values were calculated from 1000 trees. The corresponding nucleotide sequences of *Escherichia coli* (NC_013364) were used as outgroups. The scale bar indicates the estimated number of substitutions per 50 bases. All names and accession numbers are given as cited in the GenBank database

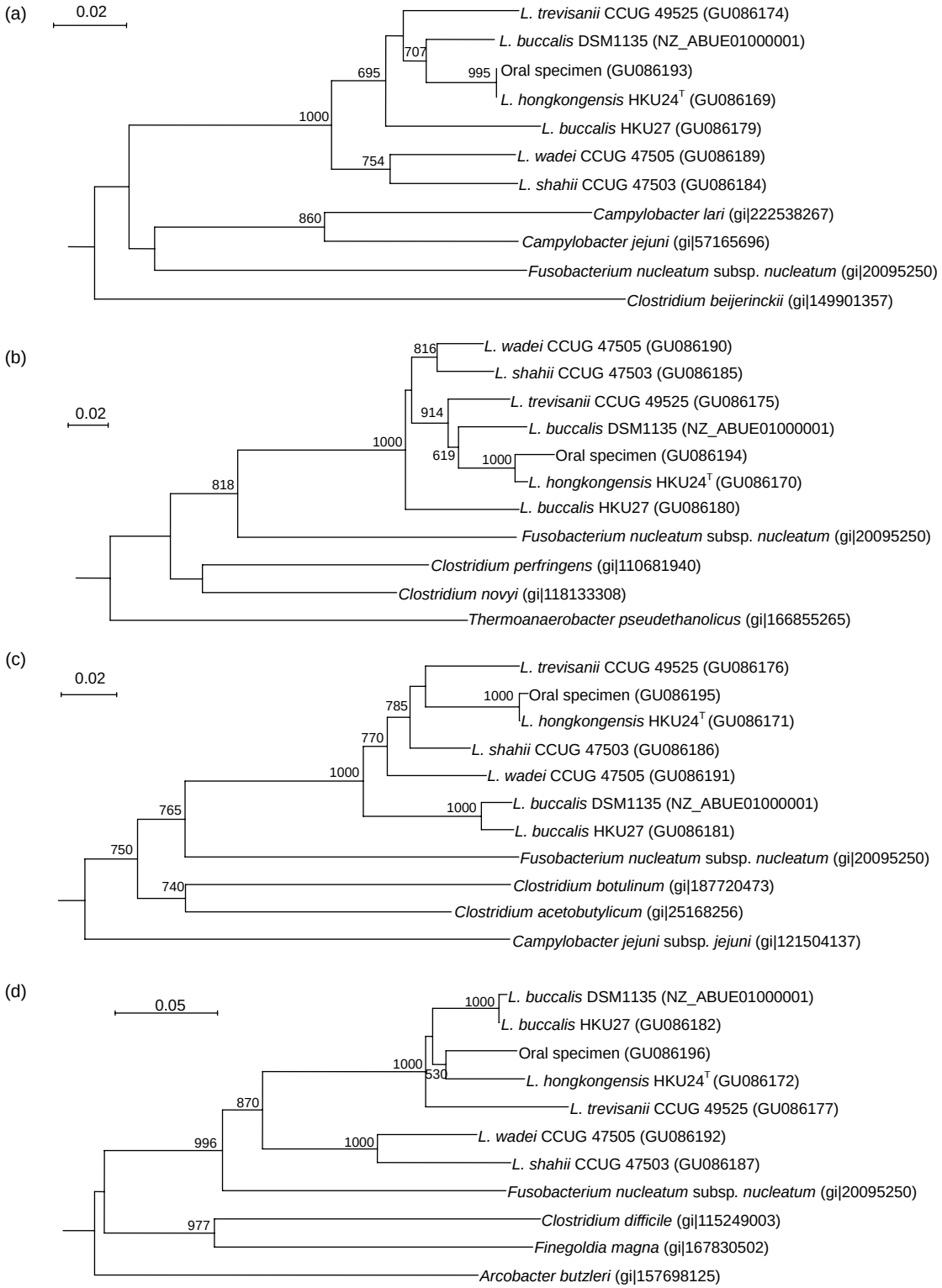

Fig. 4 Phylogenetic relationships of *groEL* (a), *gyrB* (b), *recA* (c), and *rpoB* (d) genes between the *L. hongkongensis* amplified from the oral specimen of the healthy volunteer in the present study and other *Leptotrichia* species. The trees were constructed by the neighbor-joining method and bootstrap values were calculated from 1000 trees. The corresponding nucleotide sequences of *Escherichia coli* (NC_013364) were used as outgroups. The scale bar indicates the estimated number of substitutions per 20 or 50 bases as indicated. All names and accession numbers are given as cited in the GenBank database

4 Discussion

We describe a case of *Leptotrichia* bacteremia in a non-neutropenic patient with widely disseminated carcinoma of the breast and propose a novel species, named *L. hongkongensis*, in the *Leptotrichia* genus. The clinical significance of the bacterium was evident by its isolation from blood culture as pure growth, the patient's systemic inflammatory response to the bacteremia, and the prompt response to amoxicillin-clavulanate treatment. The clinical setting was also similar to those in other cases of *Leptotrichia* infection, in which the bacterium was isolated from the blood culture of an immunocompromised patient with malignancy (Eribe and Olsen, 2008; Morgenstein *et al*., 1980; Reig *et al*., 1985; Schwartz *et al*., 1995; Ulstrup and Hartzen, 2006; Weinberger *et al*., 1991). Phenotypic characterization showed that the characteristics of HKU24^T were similar to those of a bacterium isolated from the blood culture of a neutropenic 50-year-old male patient with acute myeloid leukemia on chemotherapy, also a typical clinical setting of *Leptotrichia* infection (Patel *et al*., 1999). These two strains and other species in the genus *Leptotrichia* were all non-sporulating, non-motile Gram-variable bacilli. On the other hand, unlike other *Leptotrichia* species, these two strains were catalase-negative and exhibited fastidious growth which was enhanced with a streak of *S. aureus*. 16S rRNA gene sequence analysis showed that there were only two to six base differences between the sequence of HKU24^T and that bacterium, and two other oral clones (AF287811 and AM420200), but more than 2.5% difference compared to those of all other bacteria (Fig. 2) (Paster *et al*., 2001). Due to their unique phenotypic characteristics, the high nucleotide identity of their 16S rRNA gene sequences, and the significant difference from other *Leptotrichia* species, these two strains should constitute a novel *Leptotrichia* species. Interestingly, the strain recovered previously utilized glucose and sucrose (Patel *et al*., 1999), but this was not observed in HKU24^T using both the ANI and NH panels of the Vitek system. Therefore, basic phenotypic tests and 16S rRNA gene sequencing should be used for identification of *L. hongkongensis*. Such variation in biochemical profiles is not uncommonly observed in other bacteria, such as viridans streptococci, where biochemical tests are relatively less useful for their identification (Woo *et al*., 2004b; 2004c). Collection of additional strains and performing phenotypic tests would reveal the predominant biochemical profile of the bacterium.

Based on sequence analysis of five gene loci, HKU24^T is most closely related to *L. trevisanii*. In order to determine its phylogenetic position, we sequenced four additional housekeeping genes of HKU24^T and the four most closely related *Leptotrichia* species shown by 16S rRNA gene sequence analysis. Results showed that HKU24^T is most closely related to *L. trevisanii* for the *gyrB* and *recA* genes, in additional to the 16S rRNA gene, with high bootstraps values of 955 and 965, respectively (Fig. 3). Furthermore, HKU24^T is also quite closely related to *L. trevisanii* for the *groEL* and *rpoB* genes. Interestingly, HKU24^T is mostly closely related to *L. buccalis* for the *groEL* gene, with a high bootstrap value of 831, and is also quite closely related to *L. buccalis* for the *gyrB* and *rpoB* genes (Fig. 3). This is in contrast to its relatively distant relationship with *L. buccalis* in the 16S rRNA and *recA* genes (Figs. 2 and 3).

The oral cavity is the natural reservoir of *L. hongkongensis*. *L. buccalis* is a member of the human oral flora (Duperval *et al*., 1984; Weinberger *et al*., 1991). It causes infective endocarditis as a result of transient bacteremia due to dental procedures and neutropenic bacteremia as a result of mucositis (Caram *et al*., 2008; Crawford *et al*., 1974; Duperval *et al*., 1984; Eribe and Olsen, 2008; Hammann *et al*., 1993; Morgenstein *et al*., 1980; Munson *et al*., 2004; Reig *et al*., 1985; Schwartz *et al*., 1995; Sutter, 1984; Ulstrup and Hartzen, 2006; Weinberger *et al*., 1991). Among the five recently described novel *Leptotrichia* species, *L. hofstadii*, *L. shahii*, and *L. wadei* have been recovered in the human oral cavity (Eribe *et al*., 2004). As for *L. hongkongensis*, 16S rRNA gene sequence analysis showed that it is clustered with two oral clones (AF287811 and AM420200). Therefore, we used a molecular method, using multiple gene targets, to detect the presence of *L. hongkongensis* in oral specimens of healthy volunteers (Woo *et al*., 2008). Since the type strain of *L. hongkongensis* is resistant to metronidazole and levofloxacin, we used BACTEC Plus Anaerobic/F blood culture broth with these two antibiotics for selection and enrichment of *L. hongkongensis* in the oral specimens, as no *L. hongkongensis* was detected if antibiotics were not

used in the enrichment procedure (data not shown). Since no *L. hongkongensis* specific primers could be designed for the 16S rRNA gene, we used *L. hongkongensis* specific primers for the other four housekeeping genes that we used for phylogenetic analysis to screen for *L. hongkongensis* in oral specimens. All four genes could be amplified from the oral specimen collected from the same healthy volunteer, and sequencing and phylogenetic analysis confirmed that they were all *L. hongkongensis*. These showed that similar to most other *Leptotrichia* species, the oral cavity is the natural reservoir of *L. hongkongensis*.

Leptotrichia hongkongensis sp. nov. is described as follows: *Leptotrichia hongkongensis* (hong.kong. en'sis. N.L. fem. adj. *hongkongensis* of Hong Kong, where the type strain was isolated). Cells are straight, non-sporulating, Gram-variable bacilli. On primary subculture, it does not grow on sheep blood agar, chocolate agar, or brucella agar. After repeated subculturing in BACTEC Plus Anaerobic/F blood culture broth, it grows on brucella agar as non-hemolytic, pinpoint colonies after 96 h of incubation at 37 °C in anaerobic environment, as well as the aerobic environment with 5% CO_2. Growth is enhanced with a streak of *S. aureus*. It is non-motile. It dose not produce catalase. It is positive for alkaline phosphatase, β-glucosidase, and α-glucosidase. It hydrolyzes phenylphosphonate and reduces resazurin. The organism was isolated from the blood culture of a patient with disseminated carcinoma of the breast. The type strain of *L. hongkongensis* is strain HKU24^T.

5 Conclusions

L. hongkongensis is a novel *Leptotrichia* species isolated from the blood culture of a patient with metastatic breast carcinoma. Phylogenetically, *L. hongkongensis* is most closely related to *L. trevisanii*. The oral cavity is the natural reservoir of *L. hongkongensis*.

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