Brevibacterium massiliense sp. nov., isolated from a human ankle discharge

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Gram-positive, non-spore-forming rods, strain 5401308^T, were isolated from a human ankle discharge. Based on cellular morphology and the results of biochemical testing, this strain was tentatively identified as an undescribed member of the genus *Brevibacterium*. The major fatty acids were anteiso-C_{15:0} (45.3%), anteiso-C_{17:0} (19.2%) and iso-C_{15:0} (18.3%). Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that the bacterium was closely related to the type strains of *Brevibacterium mcbrellneri* (96.3% similarity) and *Brevibacterium paucivorans* (95.8%). On the basis of phenotypic data and phylogenetic inference, it is proposed that this strain represents a novel species, designated *Brevibacterium massiliense* sp. nov.; the type strain is 5401308^T (=CSUR P26^T=CIP 109422^T=CCUG 53855^T).

Members of the genus *Brevibacterium* include Gram-positive, non-spore-forming, non-branching rods and *Brevibacterium linens* is the type species of the genus (Skerman *et al.*, 1980). At the time of writing, 17 species with validly published names were included in the genus. Some of them were isolated from environmental samples, but several have been isolated from human samples: *Brevibacterium casei* (Collins *et al.*, 1983), *Brevibacterium epidermidis* (Collins *et al.*, 1983), *Brevibacterium mcbrellneri* (McBride *et al.*, 1993), *Brevibacterium otitidis* (Pascual *et al.*, 1996), *Brevibacterium luteolum* (Wauters *et al.*, 2003), *Brevibacterium paucivorans* (Wauters *et al.*, 2001) and *Brevibacterium sanguinis* (Wauters *et al.*, 2004). *Brevibacterium* species have been implicated in corneal ulcers (Ghosheh *et al.*, 2007), bacteraemia (Ulrich *et al.*, 2006), pericardial infection (Cannon *et al.*, 2005), endocarditis (Dass *et al.*, 2002), peritonitis (Antonioni *et al.*, 1997; Wauters *et al.*, 2000) and osteomyelitis in a neonate (Neumeister *et al.*, 1993).

In this report, a novel species belonging to the genus *Brevibacterium* that is closely related to *B. mcbrellneri* and *B. paucivorans* and was isolated from a human ankle discharge is described.

A 78-year-old man was admitted to the emergency unit of the Sainte-Marguerite Hospital, Marseille, France. He had been knocked over by a car and presented open dislocation

of the left ankle and a fibula fracture. The patient was treated with amoxicillin/clavulanic acid for 5 days (3 g day⁻¹). Three weeks later, a discharge was noted; the liquid was sampled and sent to our laboratory. Gram-positive bacilli were isolated and identified (API Coryne; bioMérieux) as a *Corynebacterium* species. As no phenotypic identification was obtained, genotypic identification based on 16S rRNA gene sequence comparison was performed and showed that the isolate, designated strain 5401308^T, was a representative of the genus *Brevibacterium*, but could not be ascribed to any known species.

MICs were determined according to the disc diffusion method as there are, to our knowledge, no international guidelines for the antimicrobial susceptibility testing of actinomycetes. Strain 5401308^T was susceptible to rifampicin, doxycycline, vancomycin and amoxicillin, but was resistant to trimethoprim/sulfamethoxazole, erythromycin and gentamicin.

Growth was tested at 37 °C on Columbia agar with 5% sheep blood incubated for 24 h in the presence of air or 5% CO₂ and also in anaerobic and microaerophilic atmospheres that were created using GENbag Anaer and GENbag Microaer (bioMérieux) systems, respectively. The strain was aerobic and also grew in a microaerophilic atmosphere and in the presence of 5% CO₂. Growth in an anaerobic atmosphere was weak after 24 h incubation. Growth at various temperatures (25, 30, 37, 45 and 50 °C) was tested. The size and ultrastructure of cells were determined by transmission electron microscopy. Cells were grown on trypticase soy broth for 24 h. Bacterial suspensions were pre-fixed in 5% (v/v) glutaraldehyde in phosphate buffer (Gibco) for at least 1 h at room temperature, washed in the same buffer and stained with 1% (w/v) phosphotungstic acid. Samples were examined

Abbreviation: MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight MS.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Brevibacterium massiliense* strain 5401308^T is EU868814.

Biolog diagnostic characteristics of *Brevibacterium massiliense* strain 5401308^T and related strains are available as supplementary material with the online version of this paper.

on a Morgagni 268D (Philips) electron microscope at an operating voltage of 60 kV.

Catalase activity was determined by using the ID Colour Catalase test kit (bioMérieux). Oxidase activity was assayed by applying cells to moistened discs that were impregnated with dimethyl-*p*-phenylenediamine (bioMérieux). NaCl tolerance was studied in trypticase soy broth containing NaCl at concentrations of 0–15 % (w/v).

The commercially available API ZYM, API Coryne and API Rapid ID 32A strips (bioMérieux) were used to characterize the biochemical properties of the isolate according to the manufacturer's instructions and incubation was performed at 37 °C. Phenotypic characteristics were compared to those of *B. luteolum* CIP 108129^T, *B. mcbrellneri* CIP 104342^T, *B. otitidis* CIP 105044^T and *B. paucivorans* CIP 107270^T, which were the most closely related species in terms of their 16S rRNA gene sequence similarities. Characteristic traits are presented in Table 1. Results of other tests on strain 5401308^T are given in the species description.

The biochemical characteristics of strain 5401308^T were also determined using the Biolog system. Cells were grown on 5 % sheep blood agar (bioMérieux). Bacterial suspensions were made in GP/GN sterile inoculation fluid (Biolog) and added to GP2 Microplates (Biolog) as described by the manufacturer. Incubation was carried out at 36 °C in an aerobic atmosphere for 21 h (see Supplementary Table S1, available in IJSEM Online) and read using the fully automated OmniLog system. The tests were repeated at least three times.

Preparation and determination of cellular fatty acids were carried out following the procedures given for the Sherlock Microbial identification System (MIDI). The fatty acid profile was characterized by the predominance of anteiso-

C_{15:0} (45.3 %), anteiso-C_{17:0} (19.2 %), iso-C_{15:0} (18.3 %) and iso-C_{16:0} (8.5 %), which were also found previously in *Brevibacterium* species that clustered with strain 5401308^T.

Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS protein analysis was carried out. Smear bacterial material was deposited on each spot of a polished-steel target plate. After air-drying, 1.5 µl matrix solution (saturated solution of α -cyanohydroxycinnaminic acid in 50 % aqueous acetonitrile containing 2.5 % trifluoroacetic acid) per spot was applied. MALDI-TOF MS was conducted using an Autoflex II MS (Bruker Daltonics). All spectra were recorded in linear, positive ion mode. The acceleration voltage was 20 kV. Spectra were collected as a sum of 675 shots across a spot. The manipulation was repeated 15 times for strain 5401308^T and the type strains of *B. luteolum*, *B. mcbrellneri*, *B. otitidis*, *B. paucivorans* and eight other representatives of the genus *Brevibacterium*. Preprocessing and identification steps were performed using the manufacturer's parameters. Consensus spectra were obtained. Scores obtained for the spectra of strain 5401308^T were significant (>2.2) only with those of strain 5401308^T and were <2.2 with spectra of all other strains tested, thus indicating that strain 5401308^T belongs to a novel species. A taxonomic tree was obtained by using the principal component analysis clustering method (Fig. 1). Strain 5401308^T clustered with the type strains of *B. luteolum*, *B. mcbrellneri*, *B. otitidis* and *B. paucivorans*.

Bacterial DNA was extracted using the MagNA Pure LC DNA isolation kit III (Roche) with the MagNA Pure LC instrument as described by the manufacturer. PCR amplification of the 16S rRNA gene was performed using the universal primer pair fD1 and rp2 (Weisburg *et al.*, 1991). PCR products were purified using MultiScreen PCR (Millipore) and sequencing reactions were carried out using a DNA sequencing kit (BigDye Terminator v1.1 Cycle Sequencing kit; PE Applied Biosystems) according to the manufacturer's instructions. Sequencing products were purified and electrophoresis was performed with the 3130 Genetic Analyzer (Applied Biosystems). The sequences obtained were compared with sequences deposited in GenBank by using the program BLAST through the NCBI server. Gene sequences were aligned using the multi-sequence alignment program CLUSTAL_X (1.8). Phylogenetic relationships with strains of closely related species were determined by using MEGA version 4.0 (Tamura *et al.*, 2007). Distance matrices were determined following the assumptions described by Kimura (1980) and were used to elaborate a dendrogram using the neighbour-joining method (Saitou & Nei, 1987). The maximum-parsimony algorithm was also used to infer phylogenetic analysis. Bootstrap analysis (bootstrap values were obtained for a consensus tree based on 100 randomly generated trees) was performed to investigate the stability of the trees obtained. Phylogenetic analysis demonstrated that our isolate is a member of the genus *Brevibacterium*, as it clustered with the type strains of *B. mcbrellneri* and *B. paucivorans* with the neighbour-joining method (Fig. 2). The same result

Table 1. Diagnostic traits of the type strains of different *Brevibacterium* species tested in this study

Strains: 1, *B. massiliense* 5401308^T; 2, *B. luteolum* CIP 108129^T; 3, *B. mcbrellneri* CIP 104342^T; 4, *B. otitidis* CIP 105044^T; 5, *B. paucivorans* CIP 107270^T. w, Weakly positive; +, positive; –, negative.

Test	1	2	3	4	5
Oxidase	–	–	–	–	+
Gelatin hydrolysis	–	+	w	+	–
Alkaline phosphatase	–	–	–	+	–
Esterase lipase (C8)	w	w	w	w	+
Pyrazinamidase	w	+	–	+	–
<i>N</i> -Acetyl- β -glucosaminidase	–	w	w	–	–
Arginine arylamidase	+	w	+	+	+
Phenylalanine arylamidase	+	+	+	+	–
Pyroglutamic acid arylamidase	–	+	–	+	–
Tyrosine arylamidase	+	+	–	+	–
Histamine arylamidase	w	w	w	–	w

Fig. 1. Dendrogram generated by using BIOTYPER software (version 2; Bruker Daltonics) showing the similarity of MALDI-TOF mass spectra of strain 5401308^T and representatives of the genus *Brevibacterium*.

was obtained with the maximum-parsimony algorithm. 16S rRNA gene sequence similarities (%) were determined by using CLUSTAL_X and MEGA version 4.0. Similarity values between strain 5401308^T and the type strains of *B. luteolum*, *B. otitidis*, *B. mcbrellneri* and *B. paucivorans* were 96.6, 96.6, 96.3 and 95.8 %, respectively. These values are <97 %, which is the value retained by the ad hoc committee to determine the species level, thus confirming that strain 5401308^T belongs to a novel species (Stackebrandt *et al.*, 2002). Based on results described

above, the name *Brevibacterium massiliense* sp. nov. is proposed for this novel species, with strain 5401308^T as the type strain.

Description of *Brevibacterium massiliense* sp. nov.

Brevibacterium massiliense (mas.si.li.en'se. L. neut. adj. *massiliense* of Massilia, the old Roman name for Marseille, where the type strain was isolated).

Fig. 2. Phylogenetic tree of representatives of the genus *Brevibacterium* inferred from 16S rRNA gene sequence comparison (1386 nt fragment). Numbers at nodes are proportions of 100 resamplings that support the topology shown. *Corynebacterium amycolatum* was used as the outgroup. Bar, 0.01 nt changes per nucleotide position.

Cells are aerobic, Gram-positive, non-spore-forming and non-motile. They are short, irregular, straight rods, 0.4–1.4 µm in length and 0.3–0.5 µm in diameter, as determined by electron microscopy. Catalase-positive and oxidase-negative. Grows between 25 and 45 °C, with optimal growth at 30–37 °C. After 24 h growth in 5% CO₂ on sheep blood agar, surface colonies are beige, shiny, smooth, circular and 0.2 mm in diameter. Tolerates NaCl, with good growth in 1–10% NaCl; no growth is observed in 15% NaCl. Using the API ZYM system, esterase (C4) and leucine arylamidase activities are detected. Esterase lipase (C8) activity is weakly positive. Alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are not detected. Using API Coryne, pyrazinamidase activity is weakly positive. Reduction of nitrates and hydrolysis of gelatin are negative. Pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, β-glucosidase and urease activities are negative. Negative for fermentation of all carbohydrates tested. Using API 32A, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase and serine arylamidase activities are detected. Histidine arylamidase activity is weakly positive. Urease, arginine dihydrolase, α-galactosidase, β-galactosidase, 6-phospho-β-galactosidase, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, glutamic acid decarboxylase, α-fucosidase, alkaline phosphatase, pyroglutamic acid arylamidase and glutamyl glutamic acid arylamidase activities are not detected. Mannose fermentation, raffinose fermentation, reduction of nitrates and indole production are negative.

Positive biochemical traits recovered when using the Biolog system are as follows: Tween 40, Tween 80, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-serine and 2,3-butanediol. The following traits are weakly positive: lactamide and TMP. The following traits are negative: α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, mannan, N-acetyl-D-glucosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, β-D-glucose, myo-inositol, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, salicin, sedoheptulosan, D-sorbitol, stachyose,

sucrose, D-tagatose, trehalose, turanose, xylitol, D-xylose, p-hydroxyphenylacetic acid, α-ketoglutaric acid, D-lactic acid methyl ester, D-malic acid, L-malic acid, succinamic acid, succinic acid, L-pyroglutamic acid, putrescine, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, AMP, UMP, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. The following traits are not reproducible: N-acetyl-β-D-mannosamine and D-ribose.

The fatty acid profile is characterized by the predominance of anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{16:0}.

The type strain, 5401308^T (=CSUR P26^T=CIP 109422^T=CCUG 53855^T), was isolated from a human ankle discharge sample.

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