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

Véronique Roux and Didier Raoult

Members of the genus Brevibacterium include Gram-positive, non-spar-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 ottidis (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 (Antoniou 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

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 5401308T, 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 5401308T 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 tryptose 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.
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 108129T, B. mcbrellneri CIP 104342T, B. otitidis CIP 105044T and B. paucivorans CIP 107270T, 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 5401308T are given in the species description.

The biochemical characteristics of strain 5401308T 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-C15 : 0 (45.3 %), anteiso-C17 : 0 (19.2 %), iso-C15 : 0 (18.3 %) and iso-C16 : 0 (8.5 %), which were also found previously in Brevibacterium species that clustered with strain 5401308T.

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-cyanohydroxycinnamic 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 5401308T 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 5401308T were significant (>2.2) only with those of strain 5401308T and were <2.2 with spectra of all other strains tested, thus indicating that strain 5401308T belongs to a novel species. A taxonomic tree was obtained by using the principal component analysis clustering method (Fig. 1). Strain 5401308T 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 (Weihsurg 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. mbrellneri 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

<table>
<thead>
<tr>
<th>Test</th>
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<th>2</th>
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<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Gelatin hydrolysis</td>
<td>–</td>
<td>+</td>
<td>w</td>
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<td>–</td>
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<td>Alkaline phosphatase</td>
<td>–</td>
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<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Esterase lipase (C8)</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>+</td>
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<td>Pyrazinamidase</td>
<td>w</td>
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<td>–</td>
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<td>N-Acetyl-β-glucosaminidase</td>
<td>–</td>
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<td>Arginine arylamidase</td>
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<tr>
<td>Phenylalanine arylamidase</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pyroglutamic acid arylamidase</td>
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<td>Tyrosine arylamidase</td>
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<tr>
<td>Histamine arylamidase</td>
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</tbody>
</table>
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\textsuperscript{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\textsuperscript{T} belongs to a novel species (Stackebrandt \textit{et al.}, 2002). Based on results described above, the name \textit{Brevibacterium massiliense} sp. nov. is proposed for this novel species, with strain 5401308\textsuperscript{T} as the type strain.

**Description of \textit{Brevibacterium massiliense} sp. nov.**

\textit{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).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig1.png}
\caption{Dendrogram generated by using BIOTYPER software (version 2; Bruker Daltonics) showing the similarity of MALDI-TOF mass spectra of strain 5401308\textsuperscript{T} and representatives of the genus \textit{Brevibacterium}.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2.png}
\caption{Phylogenetic tree of representatives of the genus \textit{Brevibacterium} inferred from 16S rRNA gene sequence comparison (1386 nt fragment). Numbers at nodes are proportions of 100 resamplings that support the topology shown. \textit{Corynebacterium amycolatum} was used as the outgroup. Bar, 0.01 nt changes per nucleotide position.}
\end{figure}
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, z-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 indole production are negative. Pyrrolidonyl arylamidase, aminopeptidase, α-naphthylamide and benzylpenicillinase activities are not detected. Using API Coryne, pyrazinamidase activity is weakly positive. Reduction of nitrates and indole production are negative. Pyrrolidonyl arylamidase, aminopeptidase, α-naphthylamide and benzylpenicillinase activities are not detected.

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: z-cyclohexadexrin, β-cyclohexadexrin, dextrin, glycogen, mannann, N-acetyl-D-glucosamine, amydalgin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-glucuronic acid, β-D-glucose, myo-inositol, z-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, melibiose, methyl z-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucose, methyl β-D-glucoside, methyl z-D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, salicin, sedoheptulose, D-sorbitol, stachyose, sucrose, D-tagatose, trehalose, turanose, xylitol, D-xylene, p-hydroxyphenylacetic acid, α-ketoglutaric acid, D-lactic acid methyl ester, D-malic acid, L-malic acid, succinic acid, succinic acid, L-pyroglutamic acid, putrescine, glycerol, adenosine, 2′-deoxyadenosine, inosine, thymidine, uridine, AMP, UMP, D-fructose 6-phosphate, z-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerophosphate. 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-C15:0 (anteiso-C17:0), iso-C15:0 and iso-C16:0. The type strain, 5401308T (=CSUR P26T = CIP 109422T = CCUG 53855T), was isolated from a human ankle discharge sample.

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References


