Microbacterium binotii sp. nov., isolated from human blood

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Two strains of non-spore-forming, rod-shaped, Gram-positive bacteria, CIP 101303^T and CIP 102116, were isolated from human blood in 1976 and 1977, respectively. These strains had chemotaxonomic markers that were consistent with classification in the genus *Microbacterium*, i.e. MK-10, MK-11 and MK-12 as the major menaquinones, predominant iso- and anteiso-branched cellular fatty acids, galactose, mannose and rhamnose as the cell-wall sugars and ornithine as the diamino acid in the cell-wall peptidoglycan. The DNA G + C content was 70–72 mol%. Comparative 16S rRNA gene sequence studies revealed that strains CIP 101303^T and CIP 102116 belonged to the genus *Microbacterium* and that they were related closely to *Microbacterium halotolerans*. The level of DNA–DNA relatedness showed that the two isolates represented a separate genomic species. Based on phenotypic and genotypic results, it is proposed that strains CIP 101303^T and CIP 102116 be assigned to a novel species, *Microbacterium binotii* sp. nov. The type strain is CIP 101303^T (=DSM 19164^T).

Orla-Jensen (1919) first established the genus *Microbacterium*, with only four species. At the time of writing, the genus includes 60 species. It has always been recognized that the genus *Microbacterium* is heterogeneous. Some species of the genera *Brevibacterium* (Collins *et al.*, 1983; Schumann *et al.*, 1999) and *Flavobacterium* (Imai *et al.*, 1984; Takeuchi & Hatano, 1998a) have been reclassified as species of the genus *Microbacterium*, and a comparative sequence analysis of the 16S rRNA gene revealed that the genera *Microbacterium* and *Aureobacterium* should be unified (Takeuchi & Hatano, 1998b; Behrendt *et al.*, 2001). Members of the genus *Microbacterium* have been isolated mainly from environmental habitats. Here, we describe the phenotypic and genotypic properties of a novel species of the genus *Microbacterium*, isolated from human blood.

Strains CIP 101303^T and CIP 102116 from the Collection de l'Institut Pasteur were isolated from human blood in 1976 and 1977, respectively, and identified as belonging to the genus *Microbacterium*. They were grown aerobically at 30 °C on trypto–casein–soy agar (Bio-Rad) and previously

examined by the conventional tests described by Renaud *et al.* (2007). Enzyme reactions and acid-producing abilities were studied by means of the API ZYM and API 50 CH systems (bioMérieux), respectively. These systems were used as recommended by the manufacturer. Physiological and biochemical characteristics were found to be identical for strain CIP 101303^T and strain CIP 102116 (Table 1).

CIP 101303^T and CIP 102116 were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry followed by spectral analysis using the SARAMIS software and database tools (Spectral Archive And Microbial Identification System; AnagnosTec). Mass spectral analysis of intact cells has evolved to provide an efficient tool for the identification of micro-organisms on various taxonomic levels (Fenselau & Demirev, 2001) and allows dereplication of novel isolates (Dieckmann *et al.*, 2005) and taxonomic revisions (Stackebrandt *et al.*, 2005). The methodology is based on the detection of abundant structural proteins, such as ribosomal proteins or DNA-binding proteins (Ryzhov & Fenselau, 2001; Fagerquist *et al.*, 2005), generating taxon-specific patterns (Valentine *et al.*, 2005). Various mathematical methods have been developed to compare mass spectra and to construct trees based on pattern similarity (Jarman & Wahl, 2006). Fresh cells were transferred directly from agar plates to a MALDI-TOF mass spectro-

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

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains CIP 101303^T and CIP 102116 are EF567306 and EU600175, respectively.

Table 1. Phenotypic characteristics of strains CIP 101303^T, CIP 102116 and *M. halotolerans* CIP 108071^T

Strains: 1, CIP 101303^T; 2, CIP 102116; 3, *M. halotolerans* CIP 108071^T. Data are from this study. +, Positive; –, negative.

Characteristic	1	2	3
Colony colour	Yellow	Yellow	White
Starch hydrolysis	+	+	–
Enzyme activity			
α -Galactosidase	+	+	–
<i>N</i> -Acetyl- β -glucosaminidase	+	+	–
Acid production from:			
Glycerol	+	+	–
L-Arabinose	+	+	–
Lactose	+	+	–
Trehalose	+	+	–

meter sample plate (stainless steel, 100 wells) by using a sterile pipette tip. For extraction, the samples were mixed immediately with 1 μ l matrix solution (75 mg 2,5-dihydroxybenzoic acid ml^{–1} in 50 % acetonitrile with 0.3 % trifluoroacetic acid) and allowed to co-crystallize at room temperature. Mass spectrometric measurements were taken on a Voyager-DE PRO Workstation (Applied Biosystems) with manual data acquisition as described by Stackebrandt *et al.* (2005).

For several of the *Microbacterium* strains, series of peaks with high intensities and an intermittent distance of 128 Da were detected, indicating the presence of polyglutamine. As the presence and intensity of respective mass signals are subject to physiological conditions to a high degree, they have limited value as taxonomic biomarkers. Therefore, all mass spectra were filtered with an exclusion list containing the series of polyglutamine masses.

MALDI-TOF mass spectrometry of the *Microbacterium* strains under study yielded mass spectra with some 30–50 distinct signals in the range of 3–5 kDa. Fig. 1 shows typical mass spectra of four species, including CIP 101303^T, in which peaks of a polyglutamine compound predominate in the lower mass range. Polyglutamine was also detected in strain CIP 102116 and in the type strains of *Microbacterium thalassium* (CIP 105728^T), *Microbacterium testaceum* (CIP 104324^T), *Microbacterium aurum* (CIP 103994^T) and *Microbacterium flavescens* (CIP 102401^T) (data not shown).

Mass spectra of the *Microbacterium* strains were found to be rather variable and only a few mass signals were found with frequencies of 50 % or higher. The dendrogram (Fig. 2) resulting from the dataset shows that CIP 101303^T and CIP 102116 were separated from all other strains at a level of 20–30 % similarity, whilst the similarity between these two strains was approximately 56 %. Further investigation was consequently performed on these two strains.

The 16S rRNA genes from the two strains studied were amplified by PCR using the universal primers A,

Fig. 1. Typical MALDI-TOF mass spectra of *Microbacterium* strains. Letters above peaks indicate mass signals that are present in multiple strains. In the mass spectrum of CIP 101303^T, a series of peaks in the lower mass range could be identified as polyglutamine.

5'-AGAGTTTGATCATGGCTCAG-3' (position 8–27, *Escherichia coli* numbering system), and H, 5'-AAGGAGGTGATCCAACCGCA-3' (1541–1522) (Böttger, 1989), in a GeneAmp thermal cycler (Perkin-Elmer) with the following cycle parameters: 4 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 57 °C and 2 min at 72 °C; with a final extension step at 72 °C for 5 min. Each PCR product was purified by filtration on P100 Gel Fine (Bio-Rad) and sequenced using primers A, H and eight other sequencing primers (*E. coli* numbering system): B, 5'-CTCCTA-CGGGAGGCAGCAGT-3' (339–358); C, 5'-ACTGCTGC-CTCCCGTAGGAG-3' (358–339); D, 5'-CGTGCCAGCA-GCCGCGTAAT-3' (514–534); E, 5'-TTACCGCGGCT-GCTGGCACGT-3' (533–514); F, 5'-GATTAGATACCC-TGGTAG-3' (786–803); G, 5'-GCATGTGGTTTAATT-CGA-3' (947–964); I, 5'-TCGAATTAACCACATGC-3' (964–947); and J, 5'-AGGGTTGCGCTCGTTGCGG-3' (1115–1097). A BigDye Terminator cycle sequencing kit and ABI PRISM 3700 DNA sequencer were used according to the manufacturer's instructions (Applied Biosystems). The almost-complete sequences of the 16S rRNA gene from CIP 101303^T (1406 nt) and CIP 102116 (1365 nt) were obtained by using the script Assembler Tool Kit developed

Fig. 2. Dendrogram of *Microbacterium* strains calculated by SARAMIS based on mass spectral fingerprints in the range of 3 000–15 000 Da.

by Eric Deveau and Betina Setterblad, Groupe Logiciels et Banques de Données, Institut Pasteur.

16S rRNA gene sequence similarity was 100 % between strains CIP 101303^T and CIP 102116 and ranged from 96 to 98 % between these two strains and all of the other type strains of *Microbacterium* species under study. According to Stackebrandt & Goebel (1994), organisms displaying <97 % 16S rRNA gene sequence similarity can be considered as not belonging to the same species. However, it has been already noted that several *Microbacterium* species show <3 % similarity between their 16S rRNA gene sequences (Ko *et al.*, 2007). The characteristics that distinguish the two novel strains from related *Microbacterium* species are listed in Table 2.

Phylogenetic analysis based on the 16S rRNA gene sequences of strains CIP 101303^T, CIP 102116 and type strains of species belonging to the genus *Microbacterium*, mostly retrieved from GenBank, was performed after elimination of poor alignment and gaps by using the Gblocks method (Castresana, 2000). The sequences were aligned with CLUSTALX (Thompson *et al.*, 1997). A distance matrix was constructed by using the Jukes–Cantor algorithm with DNADIST. A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) using PHYLIP version 3.6 and viewed by using NJPLOT (Perrière & Gouy, 1996). The stability of the grouping was estimated by bootstrap analysis (100 replications) using the PHYLIP package (Felsenstein, 1989).

The phylogenetic tree constructed by the neighbour-joining method indicated that the two isolates formed a distinct cluster within the genus *Microbacterium* (Fig. 3). The resulting topologies were then investigated by using the maximum-parsimony (Felsenstein, 1989) and maximum-likelihood (Guindon & Gascuel, 2003) methods, and the two strains that could not be assigned to a described species always formed a distinct cluster, with *Microbacterium halotolerans* as the nearest phylogenetic neighbour (Fig. 3).

Chemotaxonomic analyses for strains CIP 101303^T and CIP 102116 were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und

Zellkulturen (DSMZ), Braunschweig, Germany (Table 3). The predominant menaquinones were MK-10, MK-11 and MK-12. The polar lipids comprised diphosphatidylglycerol, phosphatidylglycerol, an unknown glycolipid and unknown phospholipids. GC analysis of cellular fatty acid methyl esters was performed by using the Sherlock MIDI system (Sasser, 1990), following the protocol of the Microbial Identification System (MIDI, 1999). The major fatty acids of strains CIP 101303^T and CIP 102116 were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. The cellular fatty acid profile was consistent with those of other members of the genus *Microbacterium*. Cell-wall sugars were obtained according to the method of Whiton *et al.* (1985) and the samples were analysed by TLC on cellulose plates as described by Stanek & Roberts (1974). The cell-wall sugars were galactose, mannose and rhamnose. The amino acid composition of the cell-wall peptidoglycan was determined after hydrolysis with 4 M HCl at 100 °C for 16 h as described by Schleifer (1985). Cell-wall peptidoglycan of CIP 101303^T and CIP 102116 contained ornithine, alanine, glycine, homoserine (Hsr), glutamic acid and hydroxyglutamic acid (Hyg). From these data, it was concluded that the peptidoglycan was of type B6: B2β {Gly} [L-Hsr] D-Glu(Hyg)–Gly–D-Orn (Schleifer & Kandler, 1972). This estimated structure was that of the genus *Aureobacterium*, which is now unified with the genus *Microbacterium* (Takeuchi & Hatano, 1998b). The phylogenetic significance of peptidoglycan types in the genus *Microbacterium* has recently been demonstrated (Richert *et al.*, 2007). In conclusion, all of these chemotaxonomic results are in accordance with the description of the genus *Microbacterium* (Takeuchi & Hatano, 1998b).

The G+C content of the DNA was determined by the Identification Service of the DSMZ by reverse-phase HPLC of nucleosides according to Mesbah *et al.* (1989). The DNA G+C contents of CIP 101303^T and CIP 102116 were 71.7 and 70.4 mol%, respectively (Table 3). These correspond with those of the members of the genus *Microbacterium*, which have high DNA G+C contents of 66.5–71.6 mol% (Takeuchi & Hatano, 1998b).

DNA–DNA hybridization to determine genomic relatedness was performed by the DSMZ as described by De Ley

Table 2. Differential characteristics between strain CIP 101303^T and the type strains of its nearest phylogenetic neighbours

Species: 1, *M. binotii*; 2, *M. halotolerans*; 3, *M. trichothecenolyticum*; 4, *M. halophilum*; 5, *M. xylanilyticum*; 6, *M. hominis*; 7, *M. thalassium*; 8, *M. terrae*; 9, *M. ketosireducens*; 10, *M. flavescens*; 11, *M. laevaniformans*; 12, *M. dextranolyticum*; 13, *M. chokolatum*; 14, *M. aurantiacum*; 15, *M. kitamiense*. Data represent the type strain of each species and are from this study, Takeuchi & Hatano (1998a, b), Behrendt *et al.* (2001), Kim *et al.* (2005) and Matsuyama *et al.* (1999). +, Positive; –, negative; ND, not determined; 6dT, 6-deoxytalose; Fuc, fucose; Glc, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Colony colour	Yellow	White	Yellow	Yellow	Yellow	Yellow–white	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange
Growth at 37 °C	+	+	–	+	+	+	–	–	–	–	+	–	+	+	+
H ₂ S production	–	–	+	–	ND	+	–	+	+	+	+	+	+	+	–
Voges–Proskauer test	–	–	–	–	+	+	–	–	–	–	+	+	–	–	–
DNA G+C content (mol%)	71.7	66.5	69	67.2	69.7	71.2	69.7	70.7	69.8	66.9	68.8	68.3	69.5	70.3	69.2
Major menaquinones	MK-11, MK-12, MK-13	MK-10, MK-11	MK-12, MK-13	MK-11, MK-12, MK-13	MK-11, MK-12, MK-13	MK-11, MK-12	MK-11, MK-12	MK-13, MK-14	MK-13	MK-13, MK-14	MK-11, MK-12	MK-11, MK-12	MK-12	MK-12	MK-11, MK-12
Cell-wall diamino acid	Orn	Orn	Orn	Orn	Orn	Lys	Orn	Orn	Orn	Orn	Lys	Lys	Orn	Orn	Orn
Peptidoglycan	B2β	B2β	B2β	B2β	B2β	B1α	B2β	B2β	B2β	B2β	B1α	B1α	B2β	B2β	ND
Major cell-wall sugars	Man, Gal, Rha	Rib, Gal, Glc, Xyl*	Gal, Glc	Man, Gal, Glc	Gal, Glc	Rha, 6dT, Gal, Man	Gal, Glc	Rha, Gal, Glc	ND	Rha, Gal, Glc	Rha, Gal, Xyl, Man	6dT, Gal, Man	Rha, Gal, Man*, Xyl*	Rha, Gal, Fuc	Rha, Gal, Glc, Man

*Trace.

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences using the neighbour-joining method, showing the position of the proposed *M. binotii* strains (CIP 101303^T and CIP 102116) in relation to type strains of species in the genus *Microbacterium*. *Leucobacter komagatae* NBRC 15245^T was used as outgroup. Bootstrap values (%) are based on 100 replicates and are shown for branches with >50% bootstrap support. ■ indicates that the corresponding node was also recovered in the trees generated by the maximum-parsimony and maximum-likelihood methods (data not shown). Bar, 1 substitution per 100 nt.

et al. (1970), with consideration of the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature

controller with an *in situ* temperature probe (Varian). *M. halotolerans* CIP 108071^T was also used for this experiment. The DNA–DNA hybridization value between CIP 101303^T and CIP 102116 was 77.1% and that between CIP 101303^T and *M. halotolerans* CIP 108071^T was 22.4%. When the recommendation of a threshold value of 70% for DNA–DNA relatedness for the definition of bacterial species (Wayne *et al.*, 1987) is considered, these results suggest strongly that the two strains represent a novel species of the genus *Microbacterium*.

Thus, based on the phenotypic and genotypic characteristics of strains CIP 101303^T and CIP 102116 and the differences observed from their nearest phylogenetic neighbours (Table 2), both strains should indeed be considered as belonging to a novel species and we accordingly propose *Microbacterium binotii* sp. nov., with CIP 101303^T as the type strain.

Description of *Microbacterium binotii* sp. nov.

Microbacterium binotii (bi.no'ti.i. N.L. gen. n. *binotii* of Binot, named in honour of Dr Binot, who created the Collection de l'Institut Pasteur).

Aerobic, Gram-positive, non-motile, non-spore-forming, short rods. Colonies are yellow, circular and approximately 1–2 mm in diameter after 48 h at 30 °C. Can grow on trypto-casein-soy agar with 0–6% NaCl. Can grow at 15–37 °C. Positive for catalase. Negative for oxidase. Starch is decomposed, but gelatin and casein are not. Voges-Proskauer and indole-production tests are negative. Nitrate is not reduced to nitrite. Positive for esterase, acid phosphatase, α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase and α- and β-glucosidase activities. Negative for ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, urease, trypsin, β-glucuronidase, α-mannosidase and α-fucosidase activities. Acid is produced from glycerol, L-arabinose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, mannitol, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, starch, β-gentiobiose, turanose and gluconate. Predominant menaquinones are MK-10, MK-11 and MK-12. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. Cell-wall sugars are galactose, mannose and rhamnose and the diamino acid in the cell-wall peptidoglycan is ornithine. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol, an unknown glycolipid and unknown phospholipids. The DNA G + C content is 70–72 mol%.

The type strain is CIP 101303^T (=DSM 19164^T) and was isolated from human blood in Suresnes, France, in 1976.

Acknowledgements

We wish to thank Dr J. P. Euzéby for his help with the Latin construction of the species name. Also, the assistance of Dr Marcel Erhard and Dr Ines Streit with mass spectral analyses is acknowledged.

Table 3. Chemotaxonomic characteristics of strains CIP 101303^T, CIP 102116 and *M. halotolerans* YIM 70130^T

Strains: 1, CIP 101303^T; 2, CIP 102116; 3, *M. halotolerans* YIM 70130^T (data from Li *et al.*, 2005). Fatty acid composition data are shown as the percentage of total fatty acids. Glc, Glucose; Gal, galactose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose; –, not detected or <1 %; ND, no data available; DPG, diphosphatidylglycerol; GL, glycolipid; PG, phosphatidylglycerol; PL, phospholipid.

Characteristic	1	2	3
DNA G + C content (mol%)	71.7	70.4	66.5
Peptidoglycan	B2β	B2β	B2β
Cell-wall sugars	Gal, Man, Rha	Gal, Man, Rha	Rib, Gal, Glc, Xyl*
Cell-wall diamino acid	Orn	Orn	Orn
Major menaquinones†	MK-10, MK-11, MK-12	MK-10, MK-11, MK-12	MK-10, MK-11
Fatty acid composition			
iso-C _{15:0}	–	–	1.19
anteiso-C _{15:0}	22.42	27.33	39.91
iso-C _{16:0}	17.34	15.20	23.40
C _{16:0}	7.25	5.13	1.97
anteiso-C _{17:0}	50.65	50.44	30.07
Polar lipids†	PG, DPG, PL, GL	PG, DPG, PL, GL	ND

*Trace.

†Analyses performed by Dr B. J. Tindall, DSMZ.

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