Brevibacterium picturae sp. nov., isolated from a damaged mural painting at the Saint-Catherine chapel (Castle Herberstein, Austria)

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Three strains showing highly similar (GTG)₅-PCR patterns were isolated from a heavily damaged mural painting at the Saint-Catherine chapel (Castle Herberstein, Austria). On the basis of 16S rRNA gene sequence similarity, the strains were attributed to *Brevibacterium*, with *Brevibacterium casei* (96.7%), *Brevibacterium iodinum* (96.7%) and *Brevibacterium linens* (96.6%) as the closest related species. Chemotaxonomic data [peptidoglycan contains meso-diaminopimelic acid; mycolic acids absent; MK-8(H₂) as the major menaquinone; polar lipids phosphatidylglycerol and diphosphatidylglycerol present; anteiso-C_{15:0} and anteiso-C_{17:0} as major fatty acids] supported the affiliation of the strains to the genus *Brevibacterium*. Additional physiological and biochemical tests confirmed the taxonomic position of the strains and allowed phenotypic differentiation from *Brevibacterium* species with validly published names. The isolates from the mural painting, therefore, represent a novel species, for which the name *Brevibacterium picturae* sp. nov. is proposed, with LMG 22061^T (= DSM 16132^T) as the type strain.

After the initial description of the genus *Brevibacterium* by Breed (1953), the genus served as a depository for various Gram-positive rods later shown in chemotaxonomic studies to belong to different coryneform genera (Collins, 1992). The description of the genus was consequently emended to embrace only those species that correspond to the type species, *Brevibacterium linens*, in genus-specific characteristics (Collins *et al.*, 1980). In addition to *Brevibacterium linens*, eight other species of the genus are currently recognized: *Brevibacterium iodinum* (Collins *et al.*, 1980), *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 avium* (Pascual & Collins, 1999), *Brevibacterium paucivorans* (Wauters *et al.*, 2001) and *Brevibacterium lutescens* (Wauters *et al.*, 2003). Euzéby & Tindall (2004) have requested an opinion of the Judicial Commission to change the name of *Brevibacterium lutescens*

to *Brevibacterium luteolum*. *Brevibacterium liquefaciens* was shown to be a synonym of *Arthrobacter nicotianae* (Stackebrandt *et al.*, 1983), yet this synonymy has only recently been validated (Gelsomino *et al.*, 2004). Three other species, *Brevibacterium halotolerans*, *Brevibacterium frigoritolerans* and *Brevibacterium stationis*, have not been subjected to genetic studies and their taxonomic positions therefore remain uncertain (Jones & Keddle, 1986).

During the study of bacteria isolated from damaged mural paintings originating from three European sites, three strains were recovered from a sample of a paint layer, showing black discoloration, located in the chancel vault of the Saint-Catherine chapel of Castle Herberstein in Austria. Strain LMG 22061^T was first isolated by using R2A agar (Difco) supplemented with 1% (w/v) NaCl; strains LMG 22062 and LMG 22063 were isolated from tryptone soy agar (TSA) supplemented with 10% (w/v) NaCl. Subcultivation was done on TSA plus 10% (w/v) NaCl and marine agar (Difco). Strains were also able to grow on TSA and nutrient agar (NA; Oxoid). Salt tolerance and pH range were tested. For salt-tolerance testing, tryptone soy broth (TSB) to which 0, 5, 10 and 15% (w/v) salt had been added was used. To assess the pH range, the pH was adjusted from pH 4.0 to pH 10.0 at 0.5 unit intervals. Results were monitored after 72 h incubation at 28 °C and were scored

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 22061^T (=DSM 16132^T), LMG 22062 and LMG 22063 are AJ620364–AJ620366.

Supplementary data on fatty acid profiles are available in IJSEM Online.

as positive if the change in OD₅₅₀ was greater than 0.300. Strains grew best in TSB with the addition of 15 % (w/v) NaCl, while growth was poor in TSB without added salt. Growth was observed in the pH range 6.0–9.0, with optimal growth at pH 7.0. The standard incubation temperature was 28 °C. The growth range was tested on NA plates at different temperatures. Good growth was also observed at 20 °C; growth was weak at 15 °C. At 37 °C, only strain LMG 22062 was able to grow.

Purification of total genomic DNA was performed as described by Heyndrickx *et al.* (1996). Repetitive sequence-based PCR genomic fingerprinting was performed with the (GTG)₅ primer (Versalovic *et al.*, 1994), applying the PCR conditions described by Rademaker & de Bruijn (1997). Electrophoresis and pattern analysis were further performed as described by Heyrman *et al.* (2003). Sequence analysis was performed as described previously by Heyrman & Swings (2001). Phylogenetic trees were constructed using BioNumerics software (Applied Maths) by applying the neighbour-joining and maximum-parsimony methods on a multiple alignment similarity matrix. The stability of relationships was assessed by using a bootstrap analysis of 1000 datasets (Fig. 1). The G+C content of DNA was determined by HPLC (Mesbah *et al.*, 1989) using further specifications as given by Logan *et al.* (2000). The three strains showed nearly identical (GTG)₅ patterns, with internal Pearson correlation above 90 % (data not shown). This high genotypic similarity was further confirmed by partial 16S rRNA gene sequencing. The first 473 bp of the gene were determined and the sequence obtained for LMG 22061^T differed only by one base deletion in comparison with the identical sequences of LMG 22062 and LMG 22063. For LMG 22061^T, an almost complete 16S rRNA gene sequence (1493 bp) was determined. Sequence similarity calculations after a neighbour-joining analysis indicated that the closest relatives of strain LMG 22061^T were *Brevibacterium casei* (96.7 %), *Brevibacterium iodinum* (96.7 %), *Brevibacterium linens* (96.6 %), *Brevibacterium epidermidis* (96.2 %) and *Brevibacterium avium* (96.2 %). Sequence similarities to the other recognized species of

Brevibacterium, excluding the species with an uncertain affiliation (see above), were in the range 93.2–93.5 %. The 16S rRNA gene sequences of *Brevibacterium stationis* and *Brevibacterium halotolerans*, previously not available in the GenBank/EMBL/DDBJ databases, were also analysed and included in the phylogenetic analyses (Fig. 1). The sequence of *Brevibacterium stationis* showed 98.8 % similarity to those of the type strains of *Corynebacterium ammoniagenes* and *Corynebacterium casei*. This is in accordance with the statement of Jones & Keddle (1986), namely that *Brevibacterium stationis* is a member of the genus *Corynebacterium*. The additional statement that *Brevibacterium stationis* and *C. ammoniagenes* may even belong to the same species cannot be confirmed on the basis of the 16S rRNA gene sequence data alone. *Brevibacterium halotolerans* showed the highest sequence similarity to *Bacillus mojavensis* and *Bacillus subtilis* (respective similarities of 99.9 and 99.7 %). Attribution to the genus *Bacillus* would be in accordance with the unsaturated menaquinones with seven isoprene units being found in *Brevibacterium halotolerans* (Collins *et al.*, 1981) yet not in other members of *Brevibacterium*. *Brevibacterium frigritolerans* was not sequenced, but fatty acid analysis (performed as previously described by Heyrman *et al.*, 1999) of the type strain LMG 21659^T attributed the species to *Bacillus megaterium* (with percentage similarity to the TSBA4.0 database of 0.600–0.702), thus also confirming its previously reported uncertain taxonomic position (Jones & Keddle, 1986). The DNA G+C content (mol%) determined for strain LMG 22061^T was 63.3 %, which is in accordance with the range of the other *brevibacteria* (see Table 1). Generally recommended and accepted criteria for delineating bacterial species state that strains showing 3 % or more 16S rRNA gene sequence dissimilarity are considered representatives of separate species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Since this was the case for the investigated isolates from the mural painting, they could be attributed to a separate genospecies without additional DNA–DNA relatedness experiments.

Analysis of the fatty acid contents of the cell wall was

Fig. 1. Phylogenetic positions based on neighbour-joining of the 16S rRNA gene sequence of strain LMG 22061^T, representative of the isolates from the mural painting, among the species of *Brevibacterium*. For *Brevibacterium* species that fell outside the main cluster, the closest relatives, according to a FASTA search (Pearson & Lipman, 1988), were included in the tree. Accession numbers are given in parentheses. Bootstrap values (expressed as percentages of 1000 replications) greater than 60 % are shown at branch points. A phylogenetic tree based on the maximum-parsimony algorithm was in good accordance with this tree (data not shown).

Table 1. Differentiating characteristics of *B. picturae* sp. nov. and the other species phylogenetically attributed to *Brevibacterium*

Species: 1, *Brevibacterium picturae* sp. nov.; 2, *Brevibacterium linens* (Collins *et al.*, 1980); 3, *Brevibacterium iodinum* (Collins *et al.*, 1980); 4, *Brevibacterium casei* (Collins *et al.*, 1983); 5, *Brevibacterium epidermidis* (Collins *et al.*, 1983); 6, *Brevibacterium avium* (Pascual & Collins, 1999); 7, *Brevibacterium mcbrellneri* (McBride *et al.*, 1993); 8, *Brevibacterium otitidis* (Pascual *et al.*, 1996); 9, *Brevibacterium paucivorans* (Wauters *et al.*, 2001); 10, *Brevibacterium lutescens* (Wauters *et al.*, 2003). +, Positive; w, weakly positive; – negative; v, variable; ND, not determined; †, conflicting results between data in species description and table of differentiating characteristics.

Characteristic	1	2	3	4	5	6	7	8	9	10
Growth at:										
20 °C	+	+	+	+	+	ND	–	–†	–	+
37 °C	v	w	w	+	+	+	+	+	+	+
Oxidase	–	w	+	–	–	+	–	ND	ND	ND
Growth on 10 % NaCl	+	+*	+*	+	+	ND	–	–†	–†	+
Nitrate reduction	v	+	+	v	v	+	–	–	–	–
Pyrazinamidase	+	+	+	+	+	ND	–	+†	–	?
Pyrrolidonyl arylamidase	–	–	–	v	–	ND	–	+†	–	+
Urease	+	–*	–*	–	–	–	–	–	–	–
Lipase (C14)	–	ND	ND	ND	ND	–	ND	–	–	+
Cystine arylamidase	–	ND	ND	ND	ND	+	ND	+	–	–
Major menaquinones	MK-8(H ₂)	MK-8(H ₂)	MK-8(H ₂)	MK-8(H ₂), MK-7(H ₂)	MK-8(H ₂)	ND	ND	ND	ND	ND
G + C content (mol%) of type strain	63.3	60–64	63	66.2–67.2	63.5	ND	63.1	ND	55.8	68.8

*Data from Jones & Keddie (1986).

†Data from Wauters *et al.* (2003).

performed as described previously (Heyrman *et al.*, 1999), starting from cells grown on TSA for 48 h. Purified cell-wall preparations were obtained by the method of Schleifer & Kandler (1972). Menaquinones were extracted as described by Collins *et al.* (1977) and were analysed by HPLC (Groth *et al.*, 1997). Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). The absence of mycolic acids was demonstrated by using TLC (Minnikin *et al.*, 1975). The fatty acid profile of the three strains (given in the species description) showed a predominance of the fatty acids anteiso-C_{15:0} and anteiso-C_{17:0}, which is in accordance with those of the other species of *Brevibacterium* (available as supplementary data in IJSEM Online). Funke & Carlotti (1994) studied several *Brevibacterium* species by means of fatty acid analysis and found that anteiso-C_{15:0} and anteiso-C_{17:0} comprised more than 75 % of the total fatty acid content for all strains studied. They concluded that this feature could be applied to differentiate members of *Brevibacterium* from other Gram-positive non-fermenters at the generic level. The species *Brevibacterium mcbrellneri* (McBride *et al.*, 1993) and *Brevibacterium otitidis* (Wauters *et al.*, 2003), and the *Brevibacterium* species of uncertain taxonomic position, do not correspond with this general observation. In a clustering based on the unweighted pair group method with arithmetic averages of Euclidian distances (not including *Brevibacterium otitidis* and *Brevibacterium lutescens*, data

not shown), the isolates from mural paintings formed a tight cluster and grouped closest with the MIDI database reference of *Brevibacterium iodinum* (>99 % similarity). *Brevibacterium frigoritolerans*, *Brevibacterium halotolerans* and *Brevibacterium stationis* showed profiles deviating from those of the other *Brevibacterium* species, which is in accordance with literature data (see above) and the additional 16S rRNA gene sequence analysis performed in this study. For strain LMG 22061^T, it was shown that the cell-wall peptidoglycan contains *meso*-diaminopimelic acid, type A1γ (direct cross-linkage), that the major menaquinone is MK-8(H₂) and that mycolic acids are absent, all of which are in accordance with the genus description. Strain LMG 22061^T contains diphosphatidylglycerol and phosphatidylglycerol, as do all other *brevibacteria* tested (Jones & Keddie, 1986), plus phosphatidylinositol, one unknown phospholipid and one unknown glycolipid. The presence of phosphatidylinositol has been shown to vary with cultural conditions (Jones & Keddie, 1986).

The results of the physiological characterization are given in the species description. Cell morphology and Gram staining were examined using phase-contrast and normal light microscopy, respectively. Catalase and oxidase determinations were performed according to the methods described by Smibert & Krieg (1994). Anaerobic growth was tested in an anaerobic chamber on NA. API ZYM and API CORYNE strips (bioMérieux) were used according

to the manufacturer's instructions. The isolates from the mural painting can be differentiated from all other *Brevibacterium* species by a positive urease reaction. Further phenotypic characteristics that can be used to differentiate between the isolates from the mural painting and the other *Brevibacterium* species are shown in Table 1.

Description of *Brevibacterium picturae* sp. nov.

Brevibacterium picturae (pic.tu'rae. L. gen. n. *picturae* pertaining, or belonging, to a painting).

Cells are Gram-positive, non-motile and non-spore-forming. On NA, no clear rod-coccus cycle is observed: after 6 and 16 h growth, cells are coccoid and occur singly (0.8 and 1.0 µm in diameter, respectively); after 24 h, cells are coccoid to oval; after 72 h growth, cells are oval or short rods occurring singly or in pairs (1.0 × 1.5–4 µm). Colonies on NA are white, low-convex, slightly transparent to opaque and circular with entire margins. Optimal growth occurs between 20 and 30 °C. Growth at 15 °C is weak and no growth is observed at 10 °C. At 37 °C, growth is variable (positive only for LMG 22062). The pH range for growth is 6–9, with an optimum at pH 7. Good growth occurs at NaCl concentrations of 5, 10 and 15 % (w/v). No growth observed in an anaerobic chamber. Catalase-positive and oxidase-negative. In the API CORYNE system, pyrazinamidase, alkaline phosphatase, urease and gelatin hydrolysis are positive. Pyrrolidonyl arylamidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, aesculin (β-glucosidase) and fermentation of ribose, xylose, mannitol and glycogen are negative. The following reactions give variable results: nitrate reduction, β-galactosidase and acid production from glucose, maltose, lactose and sucrose. Of the additional tests in the API ZYM system, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase are weakly positive. Leucine arylamidase and acid phosphatase are variable (if positive, weak). Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. The type strain is positive for glucose fermentation, weakly positive for acid phosphatase and negative for nitrate reduction, β-galactosidase, leucine arylamidase and acid production from maltose, lactose and sucrose. The major fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0} (present at about 60 and 27 %, respectively). Other fatty acids present at levels of at least 1 % are iso-C_{15:0} (± 5 %), iso-C_{16:0} (± 3 %), anteiso-C_{17:1A} (± 3 %) and iso-C_{17:0} (± 1 %). The type strain shows the following chemotaxonomic characteristics: the diamino acid of the peptidoglycan is *meso*-diaminopimelic acid; the major menaquinone is MK-8(H₂); mycolic acids are absent; and the polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, an unknown phospholipid and an unknown glycolipid.

Isolated from a mural painting discoloured by microbial growth. The G + C content is 63.3 mol% for the type strain, strain LMG 22061^T (= DSM 16132^T).

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