Paenibacillus sepulcri sp. nov., isolated from biodeteriorated mural paintings in the Servilia tomb

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In 2001, a Gram-variable, facultatively anaerobic, endospore-forming bacterium isolated from biodeteriorated mural paintings in the Servilia tomb of the Roman necropolis of Carmona was deposited as *Paenibacillus* strain LMG 19508. Subsequently, the strain was characterized in detail using phenotypic and molecular methods. The 16S rRNA gene sequence confirmed that the strain belongs to the genus *Paenibacillus* and indicated its relationship to *Paenibacillus mendelii* CCM 4839^T (96.7% sequence similarity). The predominant menaquinone was MK-7. The cell wall contained meso-diaminopimelic acid of the A1γ type. The DNA G + C content (50 mol%) and the major fatty acid (anteiso-C_{15:0}) of strain LMG 19508^T were also consistent with its affiliation to the genus *Paenibacillus*. DNA–DNA hybridization distinguished strain LMG 19508^T from other phylogenetically related *Paenibacillus* species. Therefore, the isolate represents a novel species, for which the name *Paenibacillus sepulcri* sp. nov. is proposed. The type strain is CCM 7311^T (=LMG 19508^T).

The genus *Paenibacillus* was first proposed by Ash *et al.* (1993) and its description was emended by Shida *et al.* (1997). The genus *Paenibacillus* is under intensive taxonomic investigation, with ten species having been formally described in the last year. Heyrman & Swings (2001) isolated a strain, originally classified as *Paenibacillus* sp. LMG 19508, from biodeteriorated mural paintings in the Servilia tomb at the Roman necropolis of Carmona in Seville, Spain. Thereafter, the strain was deposited in the Czech Collection of Microorganisms as CCM 7311 and used for biodiversity studies. The phylogenetic position and characteristics of this strain were investigated using a polyphasic approach. The sequence of its 16S rRNA gene showed that the closest phylogenetic neighbour is *Paenibacillus mendelii* (Šmerda *et al.*, 2005). On the basis of phylogenetic, DNA relatedness, chemotaxonomic and phenotypic analyses, we propose the classification of strain CCM 7311^T as the type strain of a novel species within the genus *Paenibacillus*.

Strain CCM 7311^T and the reference cultures listed in Table 1 were grown on tryptone soya agar (Oxoid) at 30 °C to investigate their morphological and physiological characteristics. For cellular fatty acid analysis, cells were grown on trypticase soya broth (BBL) solidified with agar (Difco) at 28 °C for 24 h. For DNA extraction, all samples were cultivated on tryptone soya agar (Oxoid) at 30 °C for 4 days.

Investigations of cell morphology, physiology and biochemistry were performed using the methods of Gordon *et al.* (1973). The method of Páčová & Kocur (1984) was used to test for the hydrolysis of Tween 80 and gelatin, and OXItest and the HIPPURATetest (Pliva-Lachema) were used for oxidase and hippurate hydrolysis tests. Anaerobic growth was determined in BBL anaerobic agar tubes (Becton Dickinson) according to the manufacturer's instructions. Acid production from carbohydrates was determined using API 50 CH strips (bioMérieux), according to the manufacturer's instructions, after incubation for up to 7 days. The distinguishing phenotypic traits of strain CCM 7311^T and closely related species are shown in Table 1.

Whole-cell fatty acids from the isolate and reference cultures were extracted and analysed according to the instructions in the manual for the Microbial Identification System (MIDI, 2001). The major fatty acid in strain CCM 7311^T is anteiso-C_{15:0}, which corresponds with that of members of the genus

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The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain CCM 7311^T is DQ291142.

A extended neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences is available as a supplementary figure in IJSEM Online.

Table 1. Distinctive phenotypic characteristics of strain CCM 7311^T and phylogenetically related species of *Paenibacillus*

Strains: 1, strain CCM 7311^T; 2, *P. mendelii* CCM 4839^T; 3, *P. curdlanolyticus* CCM 4536^T; 4, *P. glycanilyticus* JCM 11221^T; 5, *P. kobensis* CCM 4537^T; 6, *P. phyllosphaerae* CCM 7310^T. All data are from this study unless indicated. +, Positive; −, negative; w, weak reaction.

Characteristic	1	2	3	4	5	6
Anaerobic growth	−	+	−	−	−	+
Oxidase	+	+	−	−	−	+
Nitrate reduction	−	−	w	−	−*	−†
Hydrolysis of:						
Starch	−	−	+	+	+	+
Hippurate	+	−	+	+	−	−
Urea	−	−	−*	+	−*	−
Acid production from:						
Glycerol	−	+	+	+	−	+
D-Arabinose	+	+	−	−	−	−
Ribose	−	+	−	+	−	−
Galactose	+	+	+	+	+	+
D-Fructose	−	+	+	−	+	+
D-Mannose	+	−	+	+	+	+
Rhamnose	+	+	−	+	−	+
Inositol	−	−	−	+	−	−
Mannitol	+	−	−	+	−	+
Methyl α-D-mannoside	−	−	−	+	−	−
Methyl α-D-glucoside	−	−	+	+	+	−
N-Acetylglucosamine	−	−	+	+	+	+
Amygdalin	−	−	+	+	−	−
Arbutin	−	−	+	+	+	−
Melibiose	+	+	+	+	+	+
Trehalose	+	+	+	+	+	−
Inulin	−	−	−	+	−	+
D-Raffinose	+	+	+	+	+	+
Starch	−	−	+	+	+	+
Glycogen	+	−	+	+	+	+
D-Lyxose	+	−	−	−	−	−
L-Fucose	+	+	−	+	−	−
Gluconate	−	−	+	+	+	−
DNA G + C content (mol%)‡	50.0	50.8 ^a	50–52 ^b	50.5 ^c	50–52 ^b	50.7 ^d

*Results differ from those in the study of Kanzawa *et al.* (1995).

†Results differ from those in the study of Rivas *et al.* (2005).

‡Data from other studies are indicated as follows: a, Šmerda *et al.* (2005); b, Shida *et al.* (1997); c, Dasman *et al.* (2002); d, Rivas *et al.* (2005).

Paenibacillus (Shida *et al.*, 1997). The fatty acid profile of the isolate is similar to those of the related reference strains (Table 2). It was possible to differentiate the novel strain from its closest phylogenetic neighbour, *P. mendelii* CCM 4839^T on the basis of the considerably smaller amounts of C_{15:0} (2.3%), iso-C_{15:0} (5.0%) and iso-C_{17:0} (1.8%) in strain CCM 7311^T or by the absence of C_{16:1ω11c}. In comparison with other paenibacilli, strain CCM 7311^T

Table 2. Fatty acid compositions (%) of strain CCM 7311^T and type strains of closely related *Paenibacillus* species

Fatty acids present in amounts less than 1.5% are omitted. Strains: 1, strain CCM 7311^T; 2, *P. mendelii* CCM 4839^T; 3, *P. curdlanolyticus* CCM 4536^T; 4, *P. glycanilyticus* JCM 11221^T; 5, *P. kobensis* CCM 4537^T; 6, *P. phyllosphaerae* CCM 7310^T. All data are from this study. ND, Not detected.

Fatty acid	1	2	3	4	5	6
Saturated fatty acids						
C _{14:0}	1.8	1.2	1.5	2.0	1.4	1.8
C _{15:0}	2.3	6.1	2.4	0.9	3.3	5.8
C _{16:0}	6.3	5.9	6.4	8.9	9.9	12.2
C _{17:0}	0.3	1.0	0.3	ND	0.6	0.8
iso-C _{14:0}	1.4	1.3	6.0	4.3	0.6	1.9
iso-C _{15:0}	5.0	13.5	4.7	1.5	3.5	5.1
iso-C _{16:0}	13.7	11.4	23.9	34.9	13.5	11.1
iso-C _{17:0}	1.8	6.1	1.7	0.6	1.3	1.6
anteiso-C _{15:0}	59.3	45.6	47.4	43.3	58.0	48.4
anteiso-C _{17:0}	6.6	7.1	3.5	3.5	7.9	6.6
Other fatty acids						
C _{16:1ω11c}	ND	0.8	0.2	ND	ND	1.6
C _{16:1ω7c} alcohol	ND	ND	1.1	ND	ND	2.0

differed, for example, by containing a larger proportion of anteiso-C_{15:0} (59.3%). Only *Paenibacillus kobensis* CCM 4537^T had a comparable fatty acid pattern.

The peptidoglycan structure was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The cell-wall peptidoglycan was of the A1γ type, containing *meso*-diaminopimelic acid as the diagnostic diamino acid. Analysis of the respiratory quinones was carried out by the DSMZ Identification Service and Dr Brian Tindall (DSMZ). MK-7 was the only menaquinone present in strain CCM 7311^T.

Genomic DNA was isolated from the bacterial culture as described by Lambert *et al.* (1998). To determine the G + C content, DNA was hydrolysed in 90% formic acid for 30 min at 140 °C (Swarts *et al.*, 1996). The hydrolysed DNA was analysed by HPLC using a Finnigan AQA mass spectrometer (ThermoQuest). The relative amounts were determined from the peak areas and coefficients of relative molar absorption. The G + C content was 50 mol%, in accordance with the overall content for members of the genus *Paenibacillus* (Shida *et al.*, 1997).

DNA–DNA hybridization was performed according to Jahnke (1994), with the modifications of Šmerda *et al.* (2005). The levels of DNA–DNA hybridization for strain CCM 7311^T with respect to *P. mendelii* CCM 4839^T, *Paenibacillus phyllosphaerae* CCM 7310^T, *Paenibacillus curdlanolyticus* CCM 4536^T, *Paenibacillus glycanilyticus* JCM 11221^T and *P. kobensis* CCM 4537^T were 16, 15, 20, 24 and 27%, respectively. According to Wayne *et al.* (1987), these levels of

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing the position of strain CCM 7311^T within the genus *Paenibacillus*. The significance of each branch is indicated by a bootstrap percentage calculated from 2000 resamplings. Bar, 0.02 nucleotide substitutions per site. An extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online.

DNA–DNA relatedness ($\leq 70\%$) support the view that strain CCM 7311^T is genomically distinct from these other species.

Amplification and sequencing of the 16S rRNA gene were performed as described by Šmerda *et al.* (2005). The resultant 16S rRNA gene sequence was aligned with reference sequences obtained from GenBank with the CLUSTAL_X 1.8 multiple alignment program (Thompson *et al.*, 1997). Evolutionary distance matrices were calculated using the algorithm of Jukes & Cantor (1969). The phylogenetic tree was constructed with the neighbour-joining method (Saitou & Nei, 1987) using the software package TREECON for Windows (Van de Peer & De Wachter, 1994). The stability of the relationships was assessed statistically by performing a bootstrap analysis based on 1000 resamplings (Felsenstein, 1985).

The phylogenetic position of the 16S rRNA gene sequence of the strain is shown in the dendrogram (Fig. 1; see also Supplementary Fig. S1 available in IJSEM Online). The closest phylogenetic neighbours of the strain CCM 7311^T are *P. mendelii* CCM 4839^T, *P. phyllosphaerae* CCM 7310^T, *P. curdolanolyticus* CCM 4536^T and *P. kobensis* CCM 4537^T. The binary similarity values of strain CCM 7311^T and the related species ranged between 96.7% (*P. mendelii* CCM 4839^T) and 93.5% (*P. glycanilyticus* JCM 11221^T). According to Stackebrandt & Goebel (1994), these levels of 16S rRNA gene similarity ($\leq 97\%$) indicate that strain CCM 7311^T is genomically distinct from these other species.

On the basis of the phenotypic, chemotaxonomic and genotypic results presented above, we consider that strain CCM 7311^T represents a novel species of the genus *Paenibacillus*, for which we propose the name *Paenibacillus sepulcri* sp. nov.

Description of *Paenibacillus sepulcri* sp. nov.

Paenibacillus sepulcri (se.pul'cri. L. gen. neut. n. *sepulcri* from a tomb, pertaining to the place of isolation of the type strain).

Cells are Gram-variable, facultatively anaerobic rods. Spores are oval and are positioned subterminally in swollen sporangia. Moderately psychrotolerant. The temperature range for growth is 10–30 °C, but no growth occurs at 37 °C; the optimum is 25 °C. Grows at pH 6.0–8.0; the optimum is between pH 7.2 and 7.4. Tolerates up to 2% NaCl, but not 3% NaCl. Colonies on nutrient agar are circular, smooth, slightly convex with complete edges and colourless. Tests positive for catalase, oxidase, lecithinase and β -galactosidase and tests negative for arginine dihydrolase, DNase, haemolysis and citrate utilization. Hydrolyses aesculin and hippurate but not casein, starch, urea, gelatin, Tween 80 or tyrosine. Acid is produced from D-arabinose, L-arabinose, D-xylose, methyl β -D-xyloside, galactose, D-glucose, D-mannose, rhamnose, mannitol, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, D-raffinose, glycogen, β -gentiobiose, D-turanose, L-fucose and 5-keto-gluconate, but not from glycerol, erythritol, ribose, L-xylose, adonitol, D-fructose, L-sorbose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, inulin, starch, xylitol, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Does not reduce nitrate to nitrite and is negative for the production of acetoin and indole. Hydrolysis of agar is not observed. The DNA G + C content is 50 mol%. The major fatty acid is anteiso-C_{15:0}. Cell-wall analysis reveals *meso*-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan. The predominant menaquinone is MK-7.

The natural habitat is unknown. The type strain, CCM 7311^T (=LMG 19508^T), was isolated from biodeteriorated mural paintings in the Servilia tomb at the Roman necropolis of Carmona in Seville, Spain.

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