Paenibacillus mendelii sp. nov., from surface-sterilized seeds of *Pisum sativum* L.

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A Gram-variable, facultatively anaerobic, endospore-forming bacterium was isolated from surface-sterilized seeds of the garden pea and characterized with phenotypic and molecular methods. A PCR with the *Paenibacillus*-specific primer PAEN515F and the 16S rRNA gene sequence indicated that strain C/2^T belongs to the genus *Paenibacillus* and is closely related to *Paenibacillus phyllosphaerae* (94.0 % sequence similarity). Strain C/2^T generated a unique phenotypic profile, in particular for the production of acid from substrates. The DNA G + C content (50.8 mol%) and the major fatty acid (anteiso-C_{15:0}) are consistent with the genus *Paenibacillus*. DNA–DNA hybridization distinguished strain C/2^T from other phylogenetically related *Paenibacillus* species and, therefore, strain C/2^T (= CCM 4839^T = LMG 23002^T) is here described as the type strain of a novel species, for which the name *Paenibacillus mendelii* sp. nov. is proposed.

The garden pea (*Pisum sativum* L.) has been one of the model organisms used for tissue culture and transformation studies at Mendel University of Agriculture and Forestry in Brno (Czech Republic). Cultivation and regeneration *in vitro* of *Pisum sativum* ‘Caesar’ were especially difficult because of frequent latent contaminations. The hazard analysis critical control point system (Leifert *et al.*, 1994) was applied to detect sources of contamination. An unknown bacterium, strain C/2^T, was detected as being a source of contamination. This strain was isolated in February 2001, from surface-sterilized seeds of the garden pea. This finding is in agreement with the observation of Leifert *et al.* (1994), who reported micro-organisms surviving the surface sterilization of plant material. Elvira-Recuenco & Van Vuurde (2000) also proved that various bacteria are abundant in pea cultivars. Characterization of the 16S rRNA gene sequence

indicated that strain C/2^T is a member of the genus *Paenibacillus* (Ash *et al.*, 1993). An analysis of 16S rRNA gene sequence similarity (<97 %) indicated that strain C/2^T differs from all currently recognized species of the genus *Paenibacillus*.

Seeds of the garden pea, *Pisum sativum* ‘Caesar’, harvested in 1999 at Agritec (a seed-producing company in Šumperk, Czech Republic), were used in the experiment. A sample of 1 kg seeds was surface sterilized for 1 min with 70 % ethanol; this was followed by vigorous shaking in 15 % household bleach (Savo – 5 % sodium hypochlorite; Bochemie) for 15 min (Saettler *et al.*, 1989). The seeds were then rinsed three times in sterile double-distilled water, ground and then soaked in sterile double-distilled water for 3 h. The supernatant was filtered through a 0.2 µm Supor membrane disc filter (Gelman). The filter discs were placed on nutrient agar plates containing 523 medium (Viss *et al.*, 1991) and/or Leifert Waites sterility test medium (Leifert *et al.*, 1994) and incubated at 27 °C in the dark for 1 week. Strain C/2^T was isolated and purified from the colonies of micro-organisms for further study.

The isolate and reference cultures were cultivated on tryptone soy agar (Oxoid) at 30 °C for the investigation of morphological and physiological characteristics. Cells used

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C/2^T is AF537343.

Fatty acid profiles, DNA relatedness data and an extended phylogenetic tree for strain C/2^T and related *Paenibacillus* species are available as supplementary material in IJSEM Online.

for cellular fatty acid analysis were grown on trypticase soy broth (BBL) solidified with agar (Difco) at 28 °C for 24 h. For DNA extraction, all samples were cultivated on tryptone soy agar at 30 °C for 4 days.

Phenotypic characterization was performed using the methods of Gordon *et al.* (1973), with two exceptions: hydrolysis of Tween 80 and gelatin was performed according to the method of Páčová & Kocur (1984) and, for oxidase and hippurate hydrolysis, the OXItest and the HIPPURATetest (Pliva-Lachema) were used. Anaerobic growth was determined in BBL anaerobic agar (Becton Dickinson). Acid production from carbohydrates was determined using API 50 CH strips (bioMérieux), according to the manufacturer's directions, after incubation for up to 7 days. The distinguishing diagnostic traits of strain C/2^T and closely related species are shown in Table 1.

For antibiotic testing of the strain, the following antibiotic discs (Sanofi Pasteur) were used: clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), tetracycline (30 µg), vancomycin (30 µg), cephalothin (30 µg) and piperacillin (100 µg).

Whole-cell fatty acids from cultures of the isolate and from reference cultures were extracted and analysed according to the instructions of the Microbial Identification System operating manual (MIDI). The major fatty acid present was anteiso-C_{15:0}, which is as expected for the genus *Paenibacillus* (Shida *et al.*, 1997). Strain C/2^T differed from the others by having considerably larger amounts of iso-C_{15:0} (13.5%) and iso-C_{17:0} (6.1%) (Table 1; details are available in Supplementary Table S1 in IJSEM Online).

Genomic DNA was isolated from the bacterial culture as described by Lambert *et al.* (1998). For measurement of the G + C content, the DNA was hydrolysed in 90% formic acid for 30 min at 140 °C (Swarts *et al.*, 1996). Hydrolysed DNA was analysed by HPLC using a Finnigan AQA mass spectrometer (ThermoQuest). Relative amounts were determined from peak areas and coefficients of relative molar absorption. The DNA G + C content was 50.8 mol%, which is in accordance with the overall content for the genus *Paenibacillus* (Shida *et al.*, 1997).

Strain C/2^T was characterized by using PCR amplification of the 16S rRNA gene with the *Paenibacillus*-specific forward primer PAEN515F (Shida *et al.*, 1997) and universal reverse primer 1377R (Shida *et al.*, 1996). The 16S rRNA gene was amplified with primers 8F (5'-AGAGTTT-GATCCTGGCTCAG-3') and 1516R (5'-GGTGATCC-AGCCGCACCTTCC-3'), using a Progene thermal cycler (Techne). The PCR product was purified with SigmaSpin post-reaction columns (Sigma-Aldrich) and ligated into the vector pCR2.1 (Invitrogen). Competent cells of *Escherichia coli* TOP10F' were transformed with a TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmids containing the insert were

Table 1. Distinctive phenotypic characteristics and significant fatty acids of *Paenibacillus mendelii* sp. nov. C/2^T and closely related *Paenibacillus* species

Strains: 1, *Paenibacillus mendelii* C/2^T; 2, *Paenibacillus curdlandolyticus* CCM 4536^T; 3, *Paenibacillus glycanilyticus* JCM 11221^T; 4, *Paenibacillus kobensis* CCM 4537^T; 5, *Paenibacillus phyllosphaerae* CCM 7310^T. All data are from this study unless indicated otherwise. +, Positive; –, negative; W, weak reaction.

Characteristic	1	2	3	4	5
Anaerobic growth	+	–	–	–	+
Oxidase	+	–	–	–	+
Nitrate reduction	–	W	–	–*	–†
Growth at pH 8.5	+	–	+	–	+
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	–	+	+	+	+
L-Fucose	+	–	+	–	–
Gluconate	–	+	+	+	–
DNA G + C content (mol%)‡	50.8	50–52 ^a	50.5 ^b	50–52 ^a	50.7 ^c
Fatty acids (%)					
iso-C _{15:0}	13.5	4.7	1.5	3.5	5.1
iso-C _{17:0}	6.1	1.7	0.6	1.3	1.6
anteiso-C _{15:0}	45.6	47.4	43.3	58.0	48.4

*Results different from those in the study by Kanzawa *et al.* (1995).

†Result different from that in the study by Rivas *et al.* (2005).

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

sequenced using an automatic DNA analyser (ABI PRISM 310; Perkin-Elmer).

The resultant 16S rRNA gene sequence was aligned with reference sequences obtained from GenBank with the

Fig. 1. Phylogenetic relationships of strain C/2^T and other closely related *Paenibacillus* species, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 0.02 nt substitutions per site. An extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online.

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 by 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 using a bootstrap analysis based on 1000 resamplings (Felsenstein, 1985).

The phylogenetic position of the 16S rRNA gene sequence of strain C/2^T is shown in a dendrogram (Fig. 1; an extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online). Strain C/2^T was clearly located within the genus *Paenibacillus*, close to the species *Paenibacillus phyllosphaerae*, *Paenibacillus curdolanolyticus* and *Paenibacillus kobensis*. The levels of 16S rRNA gene sequence similarity to these closely related species were 94.0, 94.9 and 95.7 %, respectively.

DNA–DNA hybridization was performed according to Jahnke (1994), but with the following modifications. The genomic probe of strain C/2^T was prepared using a DIG DNA labelling and detection kit (Roche) according to the manufacturer's instructions. A positively charged nylon membrane (Roche) and a BM-purple (Roche) colorimetric substrate were used. Dot blots were scanned with a CCD camera (Sony) and analysed using the software GDS 8000 (UVP). DNA relatedness values for strain C/2^T with respect to closely related species ranged from 25 % with *Paenibacillus kobensis* to 17 % with *Paenibacillus phyllosphaerae* (see Supplementary Table S2).

According to Stackebrandt & Goebel (1994) and Wayne *et al.* (1987), the levels obtained for 16S rRNA gene similarity (≤ 97 %) and DNA–DNA similarity (≤ 70 %), respectively, support the genomic distinction of strain C/2^T from other species. On the basis of the phylogenetic, chemotaxonomic and phenotypic data, strain C/2^T should be classified as the type strain of a novel species of the genus

Paenibacillus, for which we propose the name *Paenibacillus mendelii* sp. nov.

Description of *Paenibacillus mendelii* sp. nov.

Paenibacillus mendelii (men.de'li.i. N.L. gen. n. *mendelii* of Mendel, to honour J. G. Mendel, the founder of genetics).

Cells are Gram-variable rods. Aerobic or facultatively anaerobic. Spores are oval with a subterminal position in a swollen sporangium. Optimal growth occurs between 25 and 30 °C. Colonies on nutrient agar are circular, smooth, flat, bright, translucent with entire edges and about 1–2 mm in diameter. Positive tests for catalase, oxidase, lecithinase, β -galactosidase and hydrolysis of aesculin, for anaerobic growth and for growth at pH 8.5. Acid is produced from glycerol, D-arabinose, L-arabinose, ribose, D-xylose, methyl β -xyloside, galactose, D-glucose, D-fructose, rhamnose, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, D-raffinose, β -gentiobiose, D-turanose, L-fucose and 5-ketogluconate. Negative tests for production of acetoin and indole, utilization of citrate, haemolysis, arginine dihydrolase and DNase, and for hydrolysis of casein, starch, hippurate, urea, gelatin, Tween 80 and tyrosine. Nitrate is not reduced and no growth occurs at 50 °C or in 5 % NaCl. Hydrolysis of agar is not observed. Acid is not produced from erythritol, L-xylose, adonitol, D-mannose, L-sorbose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, inulin, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Sensitive to erythromycin, tetracycline, vancomycin, cephalothin and piperacillin and resistant to clindamycin and chloramphenicol. The DNA G+C content is 50.8 mol%. The major fatty acid is anteiso-C_{15:0}.

Habitat not known; the type strain, C/2^T (= CCM 4839^T = LMG 23002^T), was isolated from surface-sterilized pea seeds.

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