Paenibacillus hunanensis sp. nov., isolated from rice seeds

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Four Gram-stain-positive, aerobic or facultatively anaerobic, motile, endospore-forming, rod-shaped bacteria, designated strains FeL05^T, FeL11, Fek19 and Fek21, were isolated from seeds of hybrid rice (*Oryza sativa* L. Jinyou 611), and their taxonomic positions were determined using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences showed that the four strains were members of the genus *Paenibacillus*. They showed 95.4 % sequence similarity or less with strains of other *Paenibacillus* species. The G + C content of strain FeL05^T was found to be 53.3 mol%. Its predominant respiratory quinone was MK-7. The predominant cellular fatty acids were anteiso-C_{15:0} (61.7 %), C_{16:0} (10.9 %), iso-C_{16:0} (7.0 %), anteiso-C_{17:0} (6.7 %) and iso-C_{15:0} (5.2 %). On the basis of its phenotypic properties and phylogenetic distinctiveness, strain FeL05^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus hunanensis* sp. nov. is proposed. The type strain is strain FeL05^T (=ACCC 10718^T =CGMCC 1.8907^T =DSM 22170^T).

Paenibacillus species are aerobic or facultatively anaerobic, endospore-forming, rod-shaped bacteria and are widely distributed in nature (Slepecky & Hemphill, 1992; Claus & Berkeley, 1986). These micro-organisms are very important from industrial and economic points of view (Slepecky & Hemphill, 1992; Priest, 1977; Chung *et al.*, 2000; Weon-Taek *et al.*, 1999). Moreover, many species of the genus, including the type species, *Paenibacillus polymyxa*, have been reported as plant growth-promoting rhizobacteria (Lebuhn *et al.*, 1997; Timmusk & Wagner, 1999; Timmusk *et al.*, 1999; Helbig, 2001; Beatty & Jensen, 2002; Maes & Baeyen, 2003; von der Weid *et al.*, 2003). In this paper, we report the taxonomic characterization of four novel bacterial strains, FeL05^T, FeL11, Fek19 and Fek21, isolated from seeds of hybrid rice (*Oryza sativa* L. Jinyou 611) sampled from the China National Hybrid Rice R&D

Center, which is situated in Hunan Province. 16S rRNA gene sequencing and biochemical tests showed that these bacterial isolates constituted a novel species of the genus *Paenibacillus*.

Strain FeL05^T and FeL11 were originally isolated on plates of Luria–Bertani (LB) agar (1⁻¹: 10 g casein peptone, 5 g yeast extract, 10 g NaCl; pH 7.0), while Fek19 and Fek21 were originally isolated on plates of King's B (KB) agar (1⁻¹: 20 g proteose peptone, 1.5 g K₂HPO₄, 1.5 g MgSO₄ · 7H₂O, 15 ml glycerol; pH 7.2), both of which had been seeded with a suspension of hybrid rice seeds and incubated at 28 °C for 3 days. The seed suspension was prepared according to the following procedure, as described by Qiu *et al.* (2007). Seeds were washed with tap water, surface-sterilized with 75 % ethanol for 3 min and 2.6 % sodium hypochlorite solution for 5 min and then rinsed with sterile double-distilled water (ddH₂O). The surface-sterilized seeds were pulverized in a ceramic mortar and diluted with sterile ddH₂O using the standard dilution-plating technique. The new isolates were maintained on LB agar slants at 4 °C and as glycerol suspensions (20 %, v/v) at –20 °C. Biomass for chemotaxonomic and molecular systematic studies was prepared by growing the strains in shake flasks of LB broth at 28 °C for 1–2 days. Cells were harvested by centrifugation and washed with

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains FeL05^T, FeL11, Fek19 and Fek21 are EU741036, EU741039, EU741029 and EU741031, respectively.

Details of acid production and fatty acid profiles of the novel strains and related type strains and an electron micrograph of cells of strain FeL05^T are available as supplementary material with the online version of this paper.

ddH₂O and then freeze-dried before use in physiological and biochemical studies.

Colonial properties of the isolates were observed on LB agar. Cell morphology was examined by light microscopy and transmission electron microscopy (TEM). The presence of flagella was investigated by TEM. Gram staining was carried out using a standard procedure (Hucker & Conn, 1923). Utilization of a variety of substrates as sole carbon sources was tested using the GP2 MicroPlate Gram Positive Identification test panel (Biolog). In addition, acid-production tests were performed with the API 50CH identification system according to the instructions of the manufacturer (bioMérieux).

Purified peptidoglycan preparations were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion according to the method of Schleifer (1985). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using previously described solvent systems (Schleifer, 1985). Molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyl amino acid isobutyl esters (MacKenzie, 1987). Cellular menaquinones were extracted and purified as described by Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989). Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC and spraying with specific reagents as described by Collins & Jones (1980). Non-hydroxylated fatty acids were extracted, purified, methylated, identified and quantified by gas chromatography with the standard Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

A loop of biomass was scraped off the agar plate, suspended in 20 ml ddH₂O and lysed by boiling for 10 min and freezing for 5 min. Following centrifugation, the supernatant was used as the template for PCR. The 16S rRNA gene was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTACGACTT-3') (Lane, 1991). Amplified products were purified and cloned into vector Top10 (Tiagen) for sequence determination. Automated sequencing was performed by using an ABI Big Dye Primer cycle-sequencing ready reaction kit and Applied Biosystems 3730 DNA sequencer. The sequencing primers were SP6 (5'-ATTTAGGTGACACTATAGAATAC-3') and T7 (5'-TAATACGACTCACTATAGGG-3'). The 16S rRNA gene sequences of the four novel bacterial strains and those of other members of the genus *Paenibacillus* retrieved from GenBank were aligned using the program CLUSTAL_X version 1.8 (Thompson *et al.*, 1997). Three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Eck & Dayhoff, 1966; Fitch, 1971) and minimum-evolution (Rzhetsky & Nei, 1992) methods from MEGA4 (Tamura *et al.*, 2007), were used to infer phylogenetic evolutionary trees. Percentages of replicate trees in which the associated taxa clustered together in the

bootstrap test (1000 replicates) are shown next to branches (Felsenstein, 1985).

Preparation of genomic DNA was carried out according to the method of Marmur (1961). The G+C content of the DNA was determined using the thermal denaturation method (Marmur & Doty, 1962) with DNA from *Escherichia coli* K-12 as a control.

Comparisons with the GenBank database by BLAST search revealed that the sequences of the new isolates did not match those of any known bacterium. The bacterium with the greatest pairwise identity to strain FeL05^T was *Paenibacillus kribbensis* AM49^T (95.35%). Comparative 16S rRNA gene sequence similarity analysis revealed that the similarities among strains FeL05^T, FeL11, Fek19 and Fek21 were more than 99%. The phylogenetic tree constructed using the neighbour-joining method (Fig. 1) suggested that strains FeL05^T, FeL11, Fek19 and Fek21 are members of the genus *Paenibacillus* but represent a distinct species. Phylogenetic trees inferred by the maximum-parsimony and minimum-evolution methods showed relationships similar to those given by the neighbour-joining method (not shown).

Morphological, cultural, physiological and biochemical characteristics of strains FeL05^T, FeL11, Fek19 and Fek21 are given in the species description and in Table 1 and Supplementary Table S1 (available in IJSEM Online), which show distinctive features of the four strains as well as those of closely related species belonging to the genus *Paenibacillus*. Cell-wall peptidoglycan of strain FeL05^T

Fig. 1. Phylogenetic tree showing the relationships of the novel strains to closely related species within the genus *Paenibacillus*. The tree is based on an alignment of almost-complete 16S rRNA gene sequences and was constructed using the neighbour-joining method (Saitou & Nei, 1987). The consensus phylogenetic tree was produced following bootstrap sampling of 1000 datasets. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 0.005 substitutions per nucleotide position.

Table 1. Characteristics that differentiate strains FeL05^T, FeL11, Fek19 and Fek21 from type strains of closely related species of the genus *Paenibacillus*

Strains: 1, FeL05^T (strains FeL11, Fek19 and Fek21 gave identical results with the exception of the DNA G+C content; see text); 2, *P. kribbensis* AM49^T (data from Yoon *et al.*, 2003); 3, *P. terrae* AM141^T (Yoon *et al.*, 2003); 4, *P. rhizosphaerae* CECAP06^T (Rivas *et al.*, 2005); 5, *P. woosongensis* YB-45^T (Lee & Yoon, 2008); 6, *P. panacisoli* Gsoil 1411^T (Ten *et al.*, 2006). +, Positive or present; (+), weakly positive; –, negative or absent; +/v, Gram-variable; NR, not reported. All strains were positive for motility and catalase activity.

Characteristic	1	2	3	4	5	6
Capsules	+	NR	NR	NR	NR	NR
Gram stain	+	+/v	+/v	+	+/v	+
Oxidase	–	–	–	+	–	+
Voges–Proskauer test	+	NR	NR	+	NR	–
Nitrate reduction	+	+	+	+	–	+
Hydrolysis of:						
Casein	+	+	+	–	+	(+)
Gelatin	–	+	+	–	–	+
Starch	+	+	+	NR	+	–
Aesculin	+	+	+	NR	+	–
Tween 80	+	+	(+)	NR	–	NR
Optimum temperature (°C)	30	30–37	30	28	37	37
Growth at/in:						
50 °C	–	–	–	+	–	–
pH 5.6	+	+	+	(+)	–	+
5 % (w/v) NaCl	+	NR	NR	+	+	+
DNA G+C content (mol%)	53.3	48.0	47.0	51.3	51.7	53.9

contained *meso*-diaminopimelic acid, and it showed peptidoglycan type A1γ m-Dpm-direct (type A31, according to http://www.dsmz.de/microorganisms/main.php?content_id=35). The major polar lipids presented in strain FeL05^T were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. Traces of phosphatidylserine and an unknown phospholipid were also detected. Cellular fatty acid profiles are shown in Supplementary Table S2. The major cellular fatty acids of strain FeL05^T were anteiso-C_{15:0} (61.7%), C_{16:0} (10.9%), iso-C_{16:0} (7.0%), anteiso-C_{17:0} (6.7%) and iso-C_{15:0} (5.2%). The fatty acid profiles of the four strains were similar to those of the type strains of the *Paenibacillus* species used in this study, but there were differences in the proportions of some fatty acids. Cells of strains FeL05^T, FeL11, Fek19 and Fek21 stained Gram-positive. Capsules were observed around cells of the four strains by light microscopy and electron microscopy. The menaquinone profile, with MK-7 as the major component, is in agreement with the description of the genus *Paenibacillus*. The DNA G+C contents of strains FeL05^T, FeL11, Fek19 and Fek21 were respectively 53.3, 53.9, 52.0 and 54.9 mol%, which lie within the range of values reported for members of the genus *Paenibacillus*.

16S rRNA gene sequence similarity of less than 97% between bacterial strains indicates that they represent different species but, at 97% 16S rRNA sequence similarity or above, DNA relatedness must be used to determine whether strains belong to different species (Stackebrandt & Goebel, 1994). The bacterium with the greatest pairwise 16S rRNA gene sequence similarity to strains FeL05^T was *P. kribbensis* AM49^T (95.35% similarity), so DNA relatedness experiments with strains of known species were omitted.

In view of the combination of morphological, physiological, chemotaxonomic and phylogenetic data discussed here, it is evident that FeL05^T, FeL11, Fek19 and Fek21 belong to the genus *Paenibacillus*. Differences in some phenotypic characteristics and their phylogenetic distinctiveness distinguish the four strains from previously described *Paenibacillus* species. On the basis of the data described above, the four strains represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus hunanensis* sp. nov. is proposed.

Description of *Paenibacillus hunanensis* sp. nov.

Paenibacillus hunanensis (hu.nan.en'sis. N.L. masc. adj. *hunanensis* of Hunan, a province of China, from where the first strains were isolated).

Cells are Gram-stain-positive, aerobic or facultatively anaerobic, motile, endospore-forming, rod-shaped bacteria (2.1–3.0 × 0.7–1.0 μm). Capsules are observed by light microscopy and electron microscopy. Motile by means of peritrichous flagella (Supplementary Fig. S1). Ellipsoidal spores are formed in sporangia and in a subterminal position in cells. After 24–48 h growth on LB agar, colonies are up to 3–7 mm in diameter, circular and raised with entire edges and off-white to yellow. The optimum temperature for growth is 30 °C and the maximum growth temperature is 44 °C. Optimum growth at pH 5.0–10.0. Reactions for catalase, nitrate reduction and hydrolysis of aesculin, Tween 80, starch and casein are positive. Gelatin is not hydrolysed. Indole production and Voges–Proskauer tests are positive. Oxidase test is negative. Grows at 0–5% NaCl. The culture medium becomes more alkaline during growth. Dextrin, glycogen, *N*-acetyl-D-glucosamine, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, D-gluconic acid, α-D-glucose, α-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, thymidine, melibiose, methyl α-D-galactoside, pyruvic acid, methyl β-D-galactoside, 3-methyl D-glucose, methyl β-D-glucoside, palatinose, D-psicose, raffinose, D-ribose, salicin, D-sorbitol, stachyose, sucrose, trehalose, D-xylose, glycerol, adenosine, inosine and uridine are used as sole carbon sources for energy and growth. In the API 50CH system, when API CHB suspension medium is used, acid is produced from L-arabinose, D-ribose, D-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch,

glycogen and gentiobiose; acid is produced weakly from glycerol, inositol, methyl α -D-glucopyranoside, inulin, turanose and gluconate. Acid is not produced from erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α -D-mannopyranoside, melezitose, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, 2-ketogluconate or 5-ketogluconate. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major cellular fatty acids are anteiso-C_{15:0}, C_{16:0}, iso-C_{16:0}, anteiso-C_{17:0} and iso-C_{15:0}. The DNA G+C content of the type strain is 53.3 mol%.

The type strain, FeL05^T (=ACCC 10718^T =CGMCC 1.8907^T =DSM 22170^T), was isolated from seeds of hybrid rice (*Oryza sativa* L.) sampled from Hunan Province, China. Strains FeL11, Fek19 and Fek21 are additional strains of the species, isolated from the same source.

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