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Paenibacillus enshidis sp. nov., Isolated from the Nodules of *Robinia pseudoacacia* L.

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Abstract A Gram-positive, motile, endospore-forming, rod-shaped bacterium, designated RP-207^T, was isolated from the nodules of *Robinia pseudoacacia* L. plants planted in Enshi District, Hubei, PR China. Phylogenetic analyses based on the 16S rRNA gene sequence showed that the novel strain was affiliated to the genus *Paenibacillus*, with its closest relatives being *Paenibacillus xylanilyticus* XIL14^T (95.6 %), *Paenibacillus peoriae* DSM8320^T (95.3 %) and *Paenibacillus polymyxa* DSM 36^T (95.3 %). The DNA G+C content was 47.0 mol %. DNA–DNA hybridization value between strain RP-207^T and *P. xylanilyticus* XIL14^T was 40.1 %. The diamino acid found in the cell wall peptidoglycan was meso-diaminopimelic. The major polar lipids were phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, an unidentified amino-phospholipid and an unknown phospholipid. The predominant menaquinone was menaquinone-7 (MK-7), and the major fatty acid was anteiso-$C_{15:0}$ and $C_{16:0}$. On the basis of its physiological and biochemical characteristics and the level of DNA–DNA hybridization, strain RP-207^T is considered to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus enshidis* sp. nov. is proposed. The type strain is RP-207^T (=CCTCC AB 2013275^T = KCTC 33519^T).

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Introduction

Members of the genus *Paenibacillus* are Gram-staining-positive rods, and the Gram-staining reaction is often weak. Ellipsoidal endospores are formed and genomic DNA G+C contents range from 39 to 54 mol % [15, 19]. There were more than 150 species of this genus (http://www.bacterio.cict.fr/p/paenibacillus.html) isolated from various ecological habitats, including soils, cattle faeces, sediments and compost, warm springs, desert sand, plant rhizosphere and root nodules, food, raw and heat-treated milk, and blood cultures [1, 3, 7, 9, 12, 15, 17]. In this paper, a novel non-nodulating bacterium RP-207^T, isolated from the nodules of *Robinia pseudoacacia* L. plants planted in China, is described based on phenotypic properties, 16S rRNA gene sequences, DNA G+C content, DNA–DNA relatedness, and chemotaxonomic properties.

Materials and Methods

Strains, Cultivation and Phenotypic Characterization

The bacterial strain studied was isolated from the nodules of *R. pseudoacacia* L. plants planted in Enshi District, Hubei, PR China. Nodules were washed several times with sterile distilled water and were then surface sterilized in ethanol (95 %, v/v) for 1 min and then in sodium hypochlorite (2 %, w/v) for 10 min. Nodules were rinsed ten times with sterile distilled water and then squashed using a sterile glass rod. Homogenized nodule tissue was inoculated on modified yeast extract mannitol agar (YMA) (g L^{-1}): mannitol, 10.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; yeast extract, 1.0; agar, 15.0. Adjust pH to 7.0

with 1 M NaOH or HCl before autoclaving at 121 °C for 20 min, and the plates were incubated at 30 °C for 3 days. After isolation, single colonies were then transferred to trypticase soy agar (TSA). A bacterial strain, designated RP-207^T, was isolated and a pure culture was maintained in a glycerol suspension (15 %) at −80 °C.

The strain RP-207^T was characterized for their phenotypic and chemotaxonomic features. Colony and cell morphology, spore production and Gram stain reaction were carried out by the procedure described by Doetsch [5]. Tests for catalase, oxidase, hydrolysis of L-arginine, aesculin, casein, gelatin, starch and L-tyrosine, DNase, urease activity, nitrate reduction, citrate utilization, hydrogen disulphide production, dihydroxyacetone from glycerol and acid from carbohydrates and Voges–Proskauer (VP) reaction and indole test were analysed based on the methodologies of [2, 13]. For scanning electron microscopy, cells were rinsed triple with 10 mM phosphate-buffered saline (PBS), and then fixed in 0.2 % glutaraldehyde, dehydrated through a graded ethanol series, critical point dried and sputter coated with gold. Samples were observed under a JSM-6390LV scanning electron microscope (Japan). Growth at different pH values (5, 5.5, 6, 7, 8, 8.5 and 9), at different temperatures (10, 15, 25, 30, 37, 45 and 50 °C) and in different NaCl (1, 2, 3, and 5 %, w/v) was tested using TSA medium. All tests were carried out by incubating the cultures at 30 °C, except for investigations into the effect of temperature on growth.

Chemotaxonomic Characterization, DNA Base Composition and Genomic Relatedness

The total cellular fatty acid content was analysed by gas–liquid chromatography (GLC) as described in the MIS operating manual [10]. The composition of amino acids in the cell wall peptidoglycan was analysed on cellulose sheets according to Schleifer [18]. Cellular menaquinones were determined as described [8]. Polar lipids were analysed as previously described [11]. The DNA G+C content was determined using the method as described [20], and DNA–DNA hybridization was performed as described previously [24]. Genomic DNA was extracted and purified according to the standard methods [16]. The 16S rRNA gene sequence of the new strain was determined as described previously [22]. An almost complete (1544 bp) 16S rRNA gene sequence was obtained and compared with 16S rRNA gene sequences deposited in public databases. Amplification of the *nifH* gene was carried out and using the forward 5′-GGCTGCGATCC(CGA)AAGGCCGA(CT)TC(CGA)ACCCG-3′ and reverse 5′-CTG(GCA)GCCTTGTT(CT)TCGCGGAT(CG)GGCATGGC-3′ primers as previously described [4].

Phylogenetic Analysis

Multiple alignment and analysis of 16S rRNA gene sequences were performed using the CLUSTAL X software [23]. The phylogenetic tree was constructed using neighbour joining as well as minimum evolution and maximum parsimony methods in MEGA 5 software [21]. Bootstrap analysis was performed to assess the confidence limits of the branching.

Results and Discussion

Morphological, Physiological and Biochemical Characteristics

Strains RP-207^T were Gram positive, spore forming, rod shaped (Fig. 1). Colonies were white, round, with a smooth surface and gently convex margins (1–3 mm in diameter). Growth was observed on TSA medium with optimal temperature at 30 °C and optimal pH at 7.0. Strain RP-207^T grew in the presence of 0–3 % (w/v) NaCl, with optimal growth on medium containing 1 % (w/v) NaCl. The physiological characteristics of strain RP-207^T are summarized in the species description below and comparisons of selective characteristics with those of the type strains of closely related species are shown in Table 1.

Chemotaxonomic Characterization and DNA Base Composition

The predominant fatty acids of strain RP-207^T were $C_{16:0}$ and anteiso-$C_{15:0}$, comprising 24.54 % and 39.48 of the total,

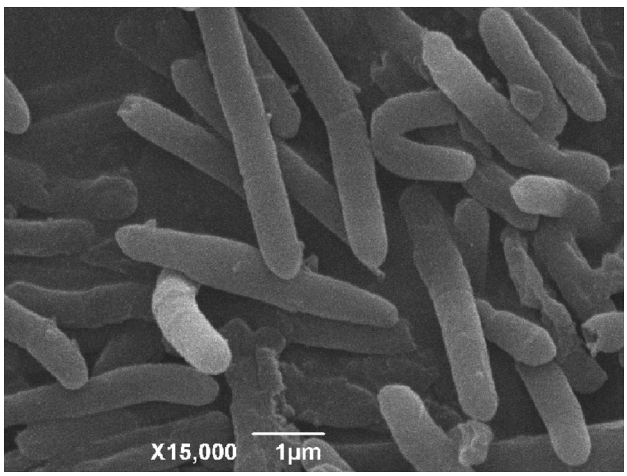

Fig. 1 Transmission electron micrograph showing general morphology of strain RP-207^T after growth for 2 days at 30 °C on RSA. *Bar* 1 μm

Table 1 Differential phenotypic characteristics of strain RP-207^T and the type strains of phylogenetically related *Paenibacillus* species

Characteristics	1	2	3	4
Spore shape	Oval	Oval	Oval	Oval
Nitrate reduction	−	−	+	+
Hydrolysis of				
Casein	+	−	+	+
Gelatin	+	+	+	+
Starch	+	+	+	+
Tween 80	+	+	+	+
Catalase	+	+	+	+
Oxidase	−	−	−	−
Utilization of				
D-Glucose	+	+	+	+
L-Arabinose	+	+	+	+
D-Fructose	+	+	+	+
D-Ribose	+	+	+	+
D-Sucrose	+	+	+	+
D-Xylose	+	+	+	+
Citrate	−	−	−	−
Acid production from				
D-Glucose	+	+	+	+
L-Arabinose	+	+	−	−
Glycerol	−	−	+	−
D-Xylose	−	+	+	+
D-Fructose	−	−	+	+
Mannitol	−	+	+	+
Sorbitol	−	−	+	−
Growth with 2 % NaCl	+	+	+	+
Growth at 50 °C	−	−	−	−
Growth at pH 5.5	−	−	+	+
Optimum growth temp. (°C)	30	37	30	30

Strains *1* strain RP-207^T (this study); *2 P. xylanilyticus* XIL14^T [14]; *3 P. polymyxa* DSM 36^T [6]; *4 Paenibacillus peoriae* DSM 8320^T. All strains tested in parallel in the present study

+ positive, − negative

Table 2 Cellular fatty acid profiles of strain RP-207^T and closely related *Paenibacillus* type strains

Fatty acid	1	2	3
Saturated straight chain			
C14:0	4.96	4.32	1.73
C15:0	ND	ND	ND
C16:0	24.54	21.84	6.89
C18:0	6.29	1.18	1.53
Saturated iso-branched			
Iso-C14:0	1.92	2.13	1.71
Iso-C15:0	4.81	5.64	3.92
Iso-C16:0	4.7	4.58	9.55
Iso-C17:0	1.88	3.86	1.97
Saturated anteiso-branched			
Anteiso-C15:0	39.48	43.20	55.05
Anteiso-C17:0	5.24	5.82	9.27
Unsaturated			
C16:1ω11c	3.37	4.85	5.96

Strains *1 P. enshidis* sp. nov. RP-207^T; *2 P. xylanilyticus* XIL14^T; *3 P. polymyxa* DSM 36^T. Data were obtained in this study. All strains were grown in TSB medium at 30 °C

respectively. The novel strain showed the same cellular fatty acid profiles as the genus *Paenibacillus*, but significant quantitative differences were observed between the novel strain described herein and related species of *Paenibacillus* (Table 2). The cell wall peptidoglycan contains meso-diaminopimelic acid. The major polar lipids were made up of phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, an unidentified amino-phospholipid and a minor amount of unknown phospholipids (Fig. S1). Menaquinone 7 (MK-7) was the major respiratory quinone in strain RP-207^T. The DNA G+C content of strain RP-207^T was 47.0 mol %.

Phylogenetic Analysis and Genomic Relatedness

The almost complete 16S rRNA gene sequence (1544 nt) of strain RP-207^T was obtained and was used for initial BLAST searches in GenBank. Strain RP-207^T showed high levels of 16S rRNA gene sequence similarity with *Paenibacillus xylanilyticus* XIL14^T (95.6 %), *P. peoriae* DSM 8320^T (95.3 %) and *P. polymyxa* DSM 36^T (95.3 %). The corresponding sequence similarities between the novel strain and the type strains of all other recognized species of the genus *Paenibacillus* were below 95.0 %. The Neighbour-joining Phylogenetic tree showing the position of the novel strain in relation to other species of the genus *Paenibacillus* is shown in Fig. 2. Similar results were obtained using the minimum evolution and maximum parsimony methods (data not shown). The result showed that strain RP-207^T formed a cluster with *P. xylanilyticus* XIL14^T within the genus *Paenibacillus*. Analysis of symbiotic genes showed that the *nifH* gene was not detected in strain RP-207^T and *P. xylanilyticus* XIL14^T. The value for DNA–DNA hybridization between strain RP-207^T and *P. xylanilyticus* XIL14^T was 40.1 %, which was clearly below the 70 % threshold value for the definition of a species.

The physiological, biochemical and phylogenetic properties of strain RP-207^T suggest that it represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus enshidis* sp. nov. is proposed.

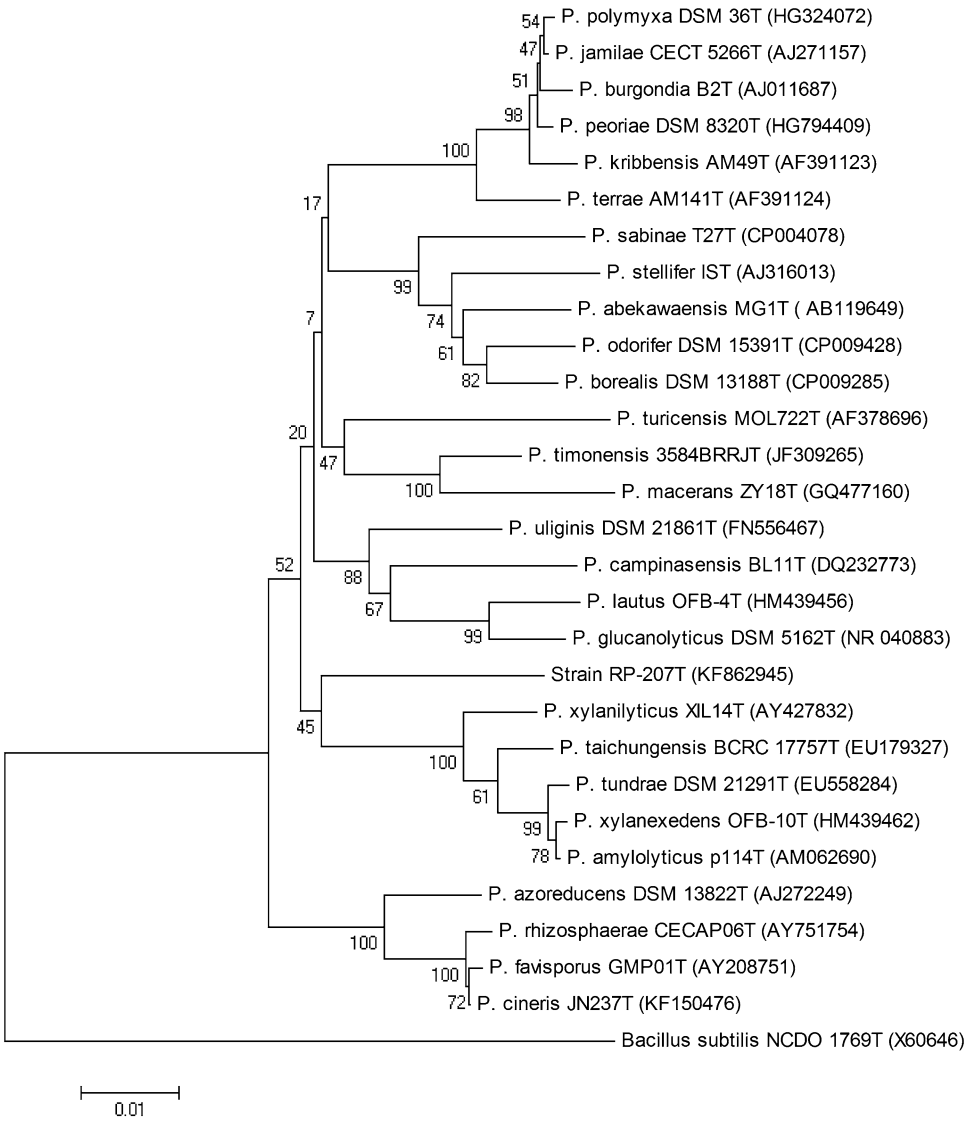

Fig. 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain RP-207^T among species of the genus *Paenibacillus*. The sequence of *Bacillus subtilis* NCDO 1769^T was used as an outgroup. *Numbers at nodes* are percentage bootstrap values based on 1000 resamplings. *Bar* 0.01 substitutions per nucleotide position

Description of *Paenibacillus enshidis* sp. nov.

Paenibacillus enshidis (en.shi'dis.N.L. masc. n. *enshidis*, pertaining to Enshi District, a district in China, from where the strain was isolated).

Cells are rod shaped with a diameter of 0.5–1 μm and a length of 3–4 μm, Gram positive, motile and strictly aerobic. Ellipsoidal spores are located centrally or subterminally in swollen sporangia. Colonies on TSA medium are circular, beige, smooth, opaque with entire margins after 72 h incubation at 30 °C and are usually 2.5–3.5 mm in diameter. The temperature range for growth is 15–45 °C, with optimal growth at 30 °C. The pH range for growth is 6–9, with optimal pH 7.0. The cells grow in 3 % (w/v) NaCl, but are unable to tolerate 5 % (w/v) NaCl. The cells are oxidase negative and catalase positive. They show negative result for indole production, H_2S formation and the Voges–Proskauer reaction, and negative result in tests for urease, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, tyrosinase and tryptophan deaminase. They hydrolyse starch, casein and gelatin, and produce acid from D-glucose, sucrose, rhamnose, L-arabinose, melibiose and D-mannose. Acids are not produced from D-xylose, glycerol, lactose, erythritol, cellobiose, maltose, D-ribose, mannitol and raffinose. This strain is able to use D-fructose, D-glucose, D-mannitol, sucrose, rhamnose, D-mannose, L-arabinose, D-ribose or D-xylose as a carbon source, but not sorbitol, citrate, inositol, malate, propionate, acetate, caprate, adipate, pyruvate or putrescine. Cell wall peptidoglycan contains meso-diaminopimelic acid. The major polar lipids were phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, an unidentified amino-phospholipid and an unknown phospholipid. The major isoprenoid quinone is MK-7. The predominant

cellular fatty acids are anteiso-$C_{15:0}$ and $C_{16:0}$. The DNA G+C content of the type strain is 47.0 mol%.

The type strain, RP-207^T (=CCTCC AB2013275^T = KCTC 33519^T), was isolated from a sample taken from the nodules of *R. pseudoacacia* L. on Enshi District in the People's Republic of China.

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