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ORIGINAL PAPER

Oceanobacillus endoradicis sp. nov., an endophytic bacterial species isolated from the root of *Paris polyphylla* Smith var. *yunnanensis*

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Abstract A bacterial strain, py1294^T, isolated from a root of *Paris polyphylla* Smith var. *yunnanensis* collected from Yunnan province, southwest China, was characterised by using a polyphasic approach to clarify its taxonomic position. Strain py1294^T was found to be Gram-positive, aerobic, spore-forming, peritrichous flagella and rod shaped. Growth was found to occur in the presence of 0–8 % (w/v) NaCl (optimum 1–3 %), at pH 6.5–9.5 (optimum 8.0) and at 10–42 °C (optimum 30 °C). The major cellular fatty acids were identified as anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and iso-$C_{14:0}$. The predominant quinone was identified as MK-7 and a minor amount of MK-6 was detected. The diagnostic polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The cell wall peptidoglycan was found to contain *meso*-diaminopimelic acid. Phylogenetic analysis of the 16S rRNA gene sequence showed that strain py1294^T forms a well-supported clade with *Oceanobacillus damuensis* PT-20^T (97.9 % sequence similarity) within the genus *Oceanobacillus*, although it also shares a high sequence similarity with *Ornithinibacillus contaminans* (97.5 %). Crucially, the DNA–DNA relatedness value between strain py1294^T and *O. damuensis* PT-20^T was 29.7 ± 3.2 %. The G+C content was determined to be 42.3 mol%. On the basis of the phylogenetic and phenotypic data, a novel species *Oceanobacillus endoradicis* sp. nov. is proposed, with py1294^T (=DSM 100726^T = KCTC 33731^T) as the type strain.

Keywords *Oceanobacillus endoradicis* sp. nov. · Polyphasic taxonomy · *Paris polyphylla* Smith var. *yunnanensis* · Endophytes

Introduction

Paris polyphylla Smith var. *yunnanensis* (Franch.) Hand.-Mazz. is a perennial medicinal plant mainly distributed in the southwest of China. The rhizomes of this plant are used in traditional Chinese medicine as a haemostatic, immunity adjustment and antimicrobial agent (Yu et al. 2010; Zhou et al. 2003). Steroidal saponins are the main active ingredient, which have important physiological activities (Waller and Yamasaki 1996). Plant endophytic microorganisms are an important source of natural bioactive compounds with great potential applications in medicine, agriculture

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and industry (Strobel and Daisy 2003; Strobel et al. 2004). Therefore, in previous work, we attempted to collect bacterial isolates from the endophytic habitat of *P. polyphylla* var. *yunnanensis*, and expect to find some useful resources, which could produce steroidal saponins or participate in the metabolism of steroidal saponins. Interestingly, amongst more than three hundred endophytic isolates, we identified an *Oceanobacillus*-like strain, py1294^T, which was isolated from a rhizome sample from Chuxiong, Yunnan province, southwest China.

The genus *Oceanobacillus* was established by Lu et al. (2001) and the description of the genus was emended by Yumoto et al. (2005), Lee et al. (2006) and Hirota et al. (2013) on the basis of chemotaxonomic and phenotypic data. At the time of writing, this genus comprises 21 validly named species, including six recently described species, *Oceanobacillus limi* (Amoozegar et al. 2014), *Oceanobacillus luteolus* (Wu et al. 2014), *Oceanobacillus arenosus* (Kim et al. 2015), *Oceanobacillus bengalensis* (Yongchang et al. 2015), *Oceanobacillus rekensis* and *Oceanobacillus damuensis* (Long et al. 2015). Members of the genus *Oceanobacillus* have been isolated from a wide variety of environments. *Oceanobacillus iheyensis*, *Oceanobacillus profundus*, *Oceanobacillus pacificus*, *O. arenosus*, *Oceanobacillus locisalsi*, *O. limi* and *O. bengalensis* were isolated from marine environments or salt lake; *Oceanobacillus kimchii*, *Oceanobacillus kapialis*, *Oceanobacillus indicireducens* and *Oceanobacillus polygoni* were isolated from fermented products (food or liquor); *O. rekensis* and *O. damuensis* were isolated from saline alkali soil; other species were isolated from fish, insect, activated sludge, mural paintings, wastewater treatment, sand dune, algal mat and soil. However, there is not yet any reports of *Oceanobacillus* species distributed in plant endophytic environments. Here, we describe an endophytic bacterial strain, py1294^T, which belongs to the genus *Oceanobacillus*.

Materials and methods

Strains and culture conditions

Healthy root samples of *P. polyphylla*. Smith var. *yunnanensis*, a traditional Chinese medicinal plant, were collected from Chuxiong, Yunnan province, southwest China (101°55.573′E 25°38.235′N), and used as sources for the isolation of endophytic bacteria. Root pieces were washed several times with running tap water and surface sterilised by stepwise washing in 5 % NaOCl for 5 min, 70 % ethanol for 8 min and finally washed several times with sterile distilled water. The dried root was pulverised in a blender, distributed on tenfold-diluted nutrient agar (10^{-1} NA) medium and incubated at 28 °C for 2 weeks. Since the growth of strain py1294^T was better on Luria–Bertani (LB) medium, for characterising this strain, it was routinely grown aerobically on LB medium for 3 days at 30 °C and pH 7.5, except where indicated otherwise. The purified strain was maintained at −80 °C on LB medium without agar and supplemented with 20 % (v/v) glycerol.

Lysinibacillus mangiferahumi M-GX18^T was obtained from Dr. Ming-He Mo (Yunnan University) for use as a reference strain during the detection of the predominant quinone. The type strain *O. damuensis* PT-20^T was kindly provided by Dr. Yongqiang Tian (Sichuan University) and cultured under comparable conditions as a reference strain.

Phenotypic characterisation

Cell morphology was examined by using phase-contrast microscopy (Leica, DM2000), transmission electron microscopy (JEOL, JEM-2100) and scanning electron microscopy (XL30 ESEM-TMP, Philips-FEI). Gram-staining was carried out according to Smibert and Krieg (1994) combined with a KOH lysis test (Gregersen 1978). Tolerance of NaCl was tested on LB plates at different NaCl concentrations (0–10 %, w/v, at intervals of 1 %). The temperature range for growth was determined at 0, 4, 10, 15, 20, 30, 37, 42 and 50 °C. Growth at various pH (4.0–10.0, at intervals of 0.5 pH units) was examined in LB liquid by using the following buffer system: pH 4.0–5.0, 0.1 mol/L citric acid/0.1 mol/L sodium citrate; pH 6.0–8.0, 0.1 mol/L KH_2PO4/0.1 mol/L NaOH; pH 9.0–10.0, 0.1 mol/L $NaHCO_3$/0.1 mol/L Na_2CO_3 (Tang et al. 2010). The pH value was measured and readjusted after autoclaving (OHAUS, STARTER3100). A spectroscopic method of monitoring turbidity at OD_{660} (Lambda 35 UV/Vis spectrometer, PerkinElmer) was used to assess the growth at various pH. Catalase activity was measured by using 3 % H_2O_2 solution. Oxidase activity was detected using

API oxidase reagent according to the manufacturer's instructions. The utilisation of carbon sources was performed using the Biolog GENIII system at 30 °C for 24 h. To determine steroid production, Liebermann-Burchard and Salkowski reactions were performed after strain py1294^T was cultured in Erlenmeyer flasks of LB liquid at 30 °C for 5 days (Zhang et al. 2007). Other physiological and biochemical characteristics, including acid production from carbohydrates were tested using the API ZYM, API 20NE and API 50CH (bioMérieux) following the manufacturers' instructions.

Chemotaxonomic characterisation

For analysis of fatty acids, cell mass of strain py1294^T and O. *damuensis* PT-20^T was harvested from tryptic soy agar (TSA; Difco) after incubation for 3 days at 30 °C. Cellular fatty acids were extracted and analysed by using the standard MIDI Sherlock Microbial Identification System (version 6.1; MIDI database: TSBA6) according to the manufacturer's instructions (Sasser 1990) on an Agilent Technologies 7890A GC. The respiratory quinones were extracted and analysed by HPLC (Agilent Technologies 1260 Infinity) (Collins et al. 1977; Groth et al. 1997), and identified by comparison with known quinones from strain *L. mangiferahumi* M-GX18^T as a reference. Total polar lipids were extracted and identified by two-dimensional TLC using the method of Minnikin et al. (1984) after staining with molybdatophosphoric acid, molybdenum blue, ninhydrin and α-naphthol. Diaminopimelic acid isomers was analysed according to the procedures developed by Hasegawa et al. (1983).

Molecular analysis

The genomic DNA was extracted and the 16S rRNA gene was amplified by PCR according to the method of Li et al. (2007). The identification of phylogenetic neighbours was achieved by using the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012). The 16S rRNA gene sequence was aligned and compared with reference sequences retrieved from the GenBank database by using CLUSTAL X software (Thompson et al. 1997). The phylogenetic trees were constructed using neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum parsimony (Fitch 1971) tree-making algorithms by using the software package MEGA version 6 (Tamura et al. 2013), while the positions with gaps were completely excluded. Distances were calculated using distance options according to Kimura's two-parameter model (Kimura 1980). The topologies of the phylogenetic trees were evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The G+C content of genomic DNA was determined using the HPLC method as described by Mesbah et al. (1989), with DNA prepared according to the method of Marmur (1961). The DNA–DNA relatedness was determined with five replications using the fluorometric microwell method (Ezaki et al. 1989) with consideration of the modifications described by Goris et al. (1998). Fluorescence was measured by using a microplate spectrofluorometer (Gemini XPS; Molecular Devices).

Results and discussion

Phenotypic characterisation

Strain py1294^T shows a range of phenotypic properties typical of members of the genus *Oceanobacillus*. It was observed to be Gram-positive, strictly aerobic, catalase and oxidase positive, and to produce terminal endospores and peritrichous flagella (Supplementary Fig. S1). Colonies were observed to be yellow, round and convex. Cells were observed to be rods, 0.3–0.6 μm wide and 2–6 μm long. Strain py1294^T was found to be able to grow at 10–42 °C, in 0–8 % (w/v) NaCl and at pH 6.5–9.5. Optimal growth was observed at 30 °C, in 1–3 % (w/v) NaCl and at pH 8.0. The strain is clearly distinct from its close neighbour *O. damuensis*, which was described as halophilic (optimum 10–15 % NaCl) and alkaliphilic (optimum pH 7.5–9.0). This difference was also found with the other closely related species listed in Table 1. Negative results from both Liebermann-Burchard and Salkowski reactions indicated that the fermentation broth of strain py1294^T did not contain steroids.

In the API 50CH strip, acid was found to be produced from D-ribose, D-fructose, *N*-acetylglucosamine, aesculin, salicin, cellobiose, starch, glycogen, D-mannitol and D-xylose. In Biolog GENIII plates, the following substrates are utilised: dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, alpha-D-lactose, D-melibiose,

Table 1 Differential properties of strain py1294^T and the type strains of closely related species of the genus *Oceanobacillus*

Characteristic	1	2	3	4	5	6
Growth temperature (°C) (optimum)	10–42 (30)	15–40 (30–37)	8–40 (30†)	8–43 (37)	5–48 (35)	14–49 (36)
pH for growth (optimum)	6.5–9.5 (8.0)	6.5–11.0 (7.5–9.0)	9.0–10.0 (9.5†)	6.0–9.0 (8.0)	7.0–12.0 (9.0)	7.0–12.0 (10.0)
NaCl tolerance (%) (optimum)	0–8 (1–3)	3–15 (10–15)	0–22 (6–12)	0.5–24 (6–14)	3–12 (3)	0–12 (10)
Nitrate reduction	−	+	+	+	+	+
Hydrolysis of						
Gelatin	+	+	w	+	−	−
Aesculin	+	−	−	−	+	+
Acid production from						
Glycerol	−	−	+	−	+	+
L-Arabinose	−	−	−	+	−	−
D-Ribose	+	−	−	−	+	+
D-Xylose	+	−	−	−	+	+
D-Fructose	+	−	−	+	+	+
D-Mannose	−	−	+	+	+	+
D-Mannitol	+	−	−	+	+	+
Salicin	+	−	−	−	+	+
Maltose	−	+	−	+	+	+
Trehalose	−	+	−	−	+	+
Melezitose	−	−	−	−	+	−
Polar lipids	DPG, PG, PE, $PL_{1,2}$, $UL_{1–3}$	DPG, PG, PE, PC, PL, $UL_{1–4}$	DPG, PG, $PL_{1–5}$, $UL^\dagger_{1,2}$	DPG, PG, PL, $GL_{1,2}$	DPG, PG, PE, $PL_{1,2}$, $UL_{1,2}$	NA
DNA G+C content (mol%)	42.3	39.2	40.0†	39.7	40.6 ± 0.9	40.0$^\$$

Strains: 1, strain py1294^T (data from this work); 2, *O. damuensis* (data from this work and Long et al. 2015); 3, *O. picturae* KCTC 3821^T; 4, *O. kapialis* KCTC 13177^T; 5, *O. polygoni* SA9^T; 6, *O. profundus* KCTC 13625^T. Data were taken from Namwong et al. (2009, columns 3, 4) and Hirota et al. (2013, columns 5, 6), except where marked († Heyrman et al. 2003; $^\$$ Kim et al. 2007)

+, Positive; −, negative; w, weakly positive; NA, not available. Urease, acid production from trehalose and melezitose were negative; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PL, unknown phospholipid; UL, unknown polar lipid; GL, unknown glycolipid

D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-beta-D-mannosamine, *N*-acetyl-D-galactosamine, alpha-D-glucose, D-fructose, D-galactose, 3-methyl glucose, L-rhamnose, inosine, D-mannitol, D-arabitol, glycerol, L-alanine, pectin, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, alpha-keto-glutaric acid, Tween 40, beta-hydroxy-DL-butyric acid, acetoacetic acid, acetic acid. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, alpha-chymotrypsin, naphthol-AS-BI-phosphohydrolase, beta-galactosidase (ONPG), hydrolysis of aesculin and gelatin are positive. Esterase lipase (C14), valine arylamidase, cystine arylamidase, acid phosphatase, alpha-galactosidase, beta-galactosidase, beta-glucuronidase, alpha-glucosidase, beta-glucosidase, *N*-acetyl-beta-glucosaminidase, alpha-mannosidase, beta-fucosidase, nitrate reduction, indole production, urease and arginine dihydrolase are negative. Phenotypic features that differentiate strain py1294^T from closely related species are summarised in Table 1.

Chemotaxonomic characterisation

Strain py1294^T was found to contain *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall, which is significantly different from that

of members of another closely related genus, *Ornithinibacillus*, which contain L-Orn–D-Asp (Mayr et al. 2006). Fatty acid composition was compared under the same experimental conditions using the close phylogenetic neighbour based on the 16S rRNA gene sequence, *O. damuensis* PT-20^T. Major fatty acids (>5 % of the total fatty acids) in strain py1294^T were identified as anteiso-$C_{15:0}$ (64.4 %), anteiso-$C_{17:0}$ (13.9 %), iso-$C_{16:0}$ (7.0 %) and iso-$C_{14:0}$ (5.2 %). $C_{16:0}$ (2.5 %) and iso-$C_{15:0}$ (4.5 %) were also detected. The presence of a large amount of anteiso-$C_{15:0}$ and the absence of $C_{18:1}$ ω9c distinguishes strain py1294^T from its close neighbour *O. damuensis* (Table 2). This fatty acid profile is similar to the description for the genus *Oceanobacillus*, but the content of each component and the absence of iso-$C_{17:0}$ were distinct from the isolate's closely related species. Polar lipid extracts were found to contain diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids and three unidentified polar lipids (Supplementary Fig. S2). The major isoprenoid quinone was found to be MK-7 (96.7 %), with MK-6 (3.3 %) present in a minor amount. Strain py1294^T exhibits chemotaxonomic profiles similar to members of the genus *Oceanobacillus*. However, the isolate could be distinguished from the closely related species of the genus *Oceanobacillus* by its fatty acid profile (Table 2) and polar lipids (Table 1).

Table 2 Cellular fatty acid compositions (%) of strain py1294^T and type strains of closely related species of the genus *Oceanobacillus*

Fatty acids	1	2	3	4	5	6
Saturated						
$C_{14:0}$	–	–	–	–	2.0	–
$C_{16:0}$	2.5	6.2	5.8	3.9	9.0	4.9
Branched chain						
Anteiso-$C_{15:0}$	64.4	42.7	52.2	47.2	60.3	53.3
Anteiso-$C_{17:0}$	13.9	17.1	14.1	21.5	8.6	18.0
iso-$C_{14:0}$	5.2	3.4	4.0	3.6	3.5	3.7
iso-$C_{15:0}$	4.5	6.1	2.3	7.4	8.3	8.5
iso-$C_{16:0}$	7.0	7.8	7.2	8.3	3.5	8.6
iso-$C_{17:0}$	–	1.6	3.8	4.0	1.3	1.9
$C_{18:1}\omega$9c	–	4.2	–	–	1.5	–
Summed feature 4	–	1.0	–	–	–	–

Strains: 1, strain py1294^T (data from this work); 2, *O. damuensis* (data from this work); 3, *O. picturae* KCTC 3821^T; 4, *O. kapialis* KCTC 13177^T; 5, *O. polygoni* SA9^T (data from Hirota et al. 2013); 6, *O. profundus* KCTC 13625^T. Values are percentages of total fatty acids; – not detected or <1.0 %. Data for columns 3, 4, 6 were taken from Lee et al. (2006). Except *O. polygoni* SA9^T, the cultural conditions and determination method for these reference strains were the same as that of strain py1294^T

Summed feature 4, $C_{17:1}$ iso I/anteiso B

Molecular analysis

The almost complete 16S rRNA gene sequence of strain py1294^T (1492 bp, GenBank/EMBL/DDBJ accession number KU189324) was determined. The 16S rRNA gene sequence displayed high levels of similarity to members of both the genera *Oceanobacillus* (between 97.9 and 93.6 %) (1354–1492 bp) and *Ornithinibacillus* (between 97.5 and 93.4 %) (1254–1491 bp) calculated by using CLUSTAL X software. High similarities are exhibited with *O. damuensis* (97.9 %, 1452 bp), *O. picturae* (96.9 %, 1492 bp), *O. kapialis* (96.8 %, 1445 bp), *O. profundus* (96.7 %, 1354 bp), *O. polygoni* (96.7 %, 1491 bp) and *Ornithinibacillus contaminans* (97.5 %, 1254 bp) respectively. Except for *O. damuensis* and *Orn. contaminans*, the similarity of 16S rRNA genes between py1294^T and other *Oceanobacillus* species are lower than 97 %. In the phylogenetic tree reconstructed using the neighbour-joining algorithm, strain py1294^T clustered with the type strain of *O. damuensis* with 98 % bootstrap support (Fig. 1). This relationship was also supported by the maximum-like and maximum-parsimony methods with high bootstrap supports of 93 and 94 % (Supplementary Fig. S3). Although strain py1294^T shows high sequence similarity with *Orn. contaminans* CCUG 53201^T, they distributed in different clades (Fig. 1a). In addition, given the close phylogenetic relationship between both genera, we generated two sequences sub-datasets, which included strain py1294^T and the members of the genera *Oceanobacillus* and *Ornithinibacillus* respectively (in each case the same outgroup, *Bacillus crassostreae*, was used). Interestingly, the phylogenetic relationship between py1294^T and the genus *Ornithinibacillus* was maintained (Fig. 1b) whilst it located outside of the genus *Ornithinibacillus* (Fig. 1c and Supplementary Fig. S3 C/F). However, based on the sequence dataset that only included the sequences of genus *Oceanobacillus*, py1294^T still clustered with *O. damuensis*, with higher bootstrapping support

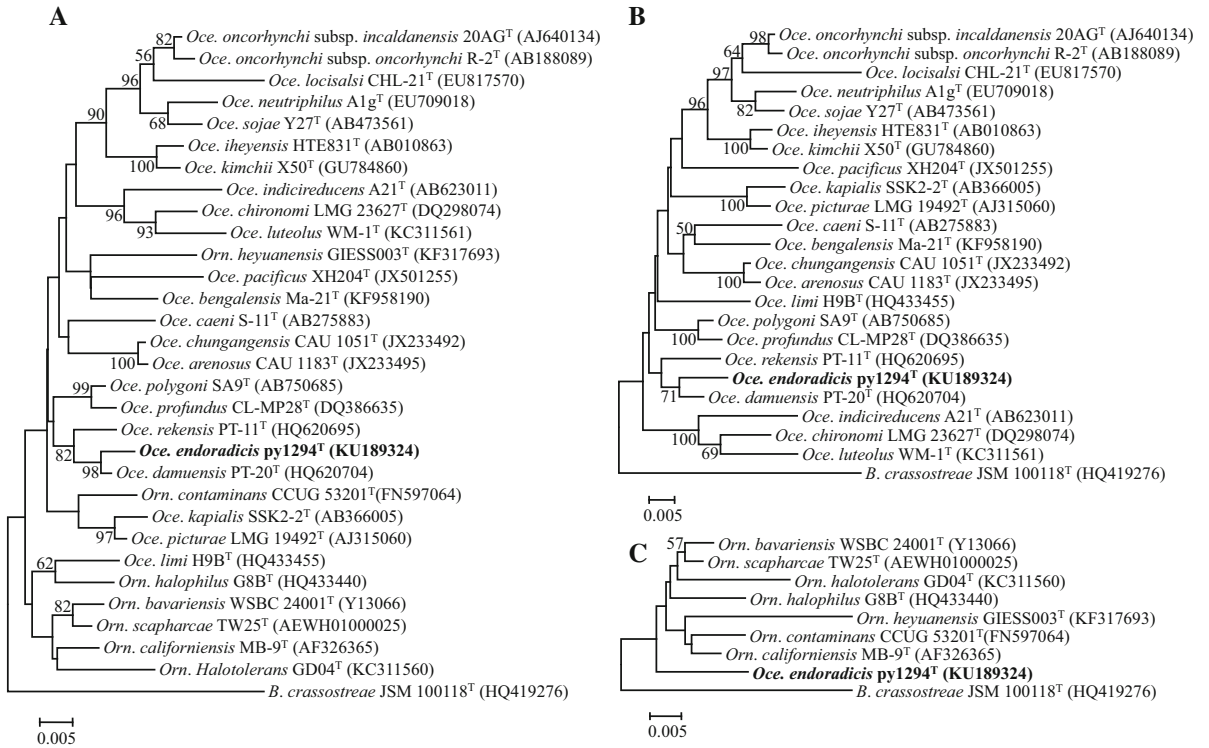

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain py1294^T. Bootstrap values (expressed as percentages of 1000 replications, and ≥50) are given at nodes. *B. crassostreae* is used as outgroup. *Bar* 0.005 substitutions per nucleotide position. *Oce.*, *Oceanobacillus*; *Orn.*, *Ornithinibacillus*; *B.*, *Bacillus*

(Fig. 1b and Supplementary Fig. S3 B/E). These results indicated that strain py1294^T belongs to the genus *Oceanbacillus*.

The DNA–DNA relatedness value between strain py1294^T and *O. damuensis* PT-20^T was 29.7 ± 3.2 %, a value that is well-below the threshold value (70 %) recommended by Wayne et al. (1987) for the definition of novel prokaryotic species.

The DNA G+C content of strain py1294^T was determined to be 42.3 mol%. Strain py1294^T shares similar chemotaxonomic characteristics with members of the genus *Oceanobacillus* in terms of the peptidoglycan type, polar lipids, predominant quinone and major fatty acids. However, strain py1294^T can be distinguished from closely related species in some chemotaxonomic and phenotypic features, such as NaCl and pH ranges for growth, and the absence of iso-$C_{17:0}$. Therefore, based on the phylogenetic analyses and the chemotaxonomic characteristics, strain py1294^T should be classified as the representative of a novel species within the genus *Oceanobacillus*, for which the name *Oceanobacillus endoradicis* sp. nov. is proposed.

Description of *Oceanobacillus endoradicis* sp. nov

Oceanobacillus endoradicis (en.do.ra′di.cis. Gr. prep. *endo* in, within; L. n. *radix—icis* a root; N.L. gen. n. *endoradicis* of the inside of a root).

Cells are Gram-positive, aerobic, catalase and oxidase positive, rod-shaped, 0.3–0.6 μm wide and 2–6 μm long. Cells produce terminal endospores and peritrichous flagella. Colonies are yellow, round, convex and 0.5–1.5 mm in diameter after incubation at 30 °C on LB for 3 days. Growth occurs at 10–42 °C (optimum 30 °C), pH 6.5–9.5 (optimum at pH 8.0) and in the presence of 0–8 % (w/v) NaCl (optimum 1–3 %). The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The major fatty acids (>5 % of the total) are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and iso-$C_{14:0}$. Polar

lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, unidentified phospholipids and unidentified polar lipids. The predominant isoprenoid quinone is MK-7. The DNA G+C content of the type strain is 42.3 mol%.

The type strain, py1294^T (=DSM 100726^T = KCTC 33731^T) was isolated from the root of *P. polyphylla*. Smith var. *yunnanensis*, collected from Chuxiong, Yunnan province, China. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain py1294^T is KU189324.

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