Paenibacillus fonticola sp. nov., isolated from a warm spring

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A novel bacterial strain, designated ZL^T, isolated from a warm spring in Jhonglun, Taiwan, was characterized by using a polyphasic taxonomic approach. The novel strain had chemotaxonomic and morphological properties consistent with its classification in the genus *Paenibacillus*. Cells were Gram-variable, aerobic, sporulating, motile rods. 16S rRNA gene sequence analysis demonstrated that this novel isolate was unique, showing 94.3% sequence similarity to *Paenibacillus assamensis* GPTSA 11^T and lower levels to *Paenibacillus timonensis* 2301032^T (94.0%), *Paenibacillus macerans* ATCC 8244^T (93.3%), *Paenibacillus barengoltzii* SAFN-016^T (93.3%) and *Paenibacillus sanguinis* 2301083^T (93.2%). The novel isolate could be distinguished from the type strains of all of these species based on a range of phenotypic data. The major cellular phospholipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and one unknown phosphoglycerolipid. The predominant isoprenologue was an unsaturated menaquinone with seven isoprene units (MK-7). The major fatty acids of strain ZL^T were C_{16:0} (33.5%), anteiso-C_{15:0} (32.5%) and iso-C_{16:0} (9.3%). The G + C content of the genomic DNA was 49.2 mol%. It is evident from the genotypic and phenotypic data that strain ZL^T should be classified as representing a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus fonticola* sp. nov. is proposed. The type strain is ZL^T (= BCRC 17579^T = LMG 23577^T).

The genus *Paenibacillus* was erected by Ash *et al.* (1993) to accommodate 11 species previously assigned to the genus *Bacillus*. Since then, many novel *Paenibacillus* species have been described. At the time of writing, more than 60 species are recognized as members of the genus (<http://www.bacterio.cict.fr/p/paenibacillus.html>). Species belonging to the genus *Paenibacillus* have been isolated from various ecological niches such as soil, rhizospheres, phyllospheres, water, insects, food, faeces, blood cultures and Antarctic sediments (Saha *et al.*, 2005). A few *Paenibacillus* species have been isolated from human biological samples and the possibility that these bacteria may cause human infection is of particular concern (Roux & Raoult, 2004). Generally,

members of this genus produce endospores, possess anteiso-C_{15:0} as the major cellular fatty acid and have genomic DNA G + C contents in the range 39–54 mol% (Shida *et al.*, 1997a; Saha *et al.*, 2005).

The aim of the present study was to determine the taxonomic position of a novel *Paenibacillus*-like organism, designated strain ZL^T, by using a polyphasic taxonomic approach.

In January 2006, strain ZL^T was isolated from a water sample collected from Jhonglun warm spring in Chiayi County, Taiwan. The temperature of the spring water ranged between 35 and 45 °C and the pH was between 7 and 8. The water sample was diluted with sterile distilled water and vortexed thoroughly. Appropriate dilutions were then plated on trypticase soy broth agar and incubated at 42 °C for 2 days. The majority of colonies (>99%) showed identical morphology.

The optimum pH range for growth was examined in trypticase soy broth by using appropriate biological buffers

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZL^T is DQ453131.

Neighbour-joining and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences showing the position of strain ZL^T within the genus *Paenibacillus* are available as supplementary figures with the online version of this paper.

(pH 3–11) (Chung *et al.*, 1995). The optimum temperature for growth (15–60 °C) of the novel strain was examined in trypticase soy broth adjusted to pH 7. Growth was examined by measuring the turbidity (OD₆₀₀) of cultures grown at various pH values and temperatures. Anaerobic cultivation was performed on nutrient agar and trypticase soy broth agar by using an Oxoid AnaeroGen system.

Strain ZL^T showed good growth on complex media, such as trypticase soy broth, nutrient agar and LB medium. Strain ZL^T formed visible, cream/white-coloured, semi-transparent colonies of a circular shape with entire edges. Colonies were approximately 0.3–0.5 mm in diameter after 48 h incubation at 42 °C. Strain ZL^T showed good growth at temperatures ranging from 20 to 45 °C and pH 7 and 10. Optimal growth was seen at 35–42 °C and pH 8.0–9.0.

Cells were observed in the lag exponential and stationary phases of growth under a phase-contrast microscope to ascertain shape and motility (using the hanging-drop technique). Flagellum staining was performed by using Spot Test Flagella Stain (BD Difco). Gram Stain Set S (BD) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction. Poly- β -hydroxybutyrate granule accumulation was observed under light microscopy after staining cells with Sudan black.

Cells of strain ZL^T stained Gram-variable and were straight rods (0.8–1.0 μ m wide and 2.0–12.4 μ m long). Spore-forming cells were rare (endospores). Terminal or sub-terminal ellipsoidal spores were observed in non-swollen or slightly swollen sporangia. Cells were motile by means of peritrichous flagella. No accumulation of poly- β -hydroxybutyrate granules was observed.

Extraction of genomic DNA, PCR amplification and sequencing of 16S rRNA genes were carried out as described by Chen *et al.* (2001). Sequence analysis was performed with a DNA sequencer (ABI PRISM 310; Applied Biosystems) and the Fragment Assembly System program from the Wisconsin Package 9.1 (Genetics Computer Group, 1995) supplied by the National Health Research Institute of Taiwan. The resultant sequence was compared with the

corresponding results taken from the EMBL, GenBank and RDP II databases for representatives of the genera classified in the order *Bacillales*. Multiple-sequence alignment of strain ZL^T with its closest relatives was performed by using BioEdit software (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. Unrooted tree topologies were evaluated by bootstrap analyses of the neighbour-joining data set (Felsenstein, 1993). An almost complete 16S rRNA gene sequence (1464 nt) was obtained for strain ZL^T. A comparison of this sequence with those of representatives of the genera classified in the order *Bacillales* showed that the novel organism fell within the evolutionary radiation occupied by the genus *Paenibacillus*. Maximum 16S rRNA gene sequence similarity between strain ZL^T and the type strains of recognized members of the genus *Paenibacillus* was 94.3 %.

It is evident from the phylogenetic tree based on the neighbour-joining algorithm that strain ZL^T clearly forms a monophyletic branch in the genus *Paenibacillus* (see Fig. 1 and Supplementary Fig. S1 available in IJSEM Online). The organism was most closely related to *Paenibacillus assamensis* GPTSA 11^T, sharing a 16S rRNA gene sequence similarity of 94.3 %. Strain ZL^T also shared comparatively low 16S rRNA gene sequence similarity with *Paenibacillus timonensis* 2301032^T (94.0 %), *Paenibacillus macerans* ATCC 8244^T (93.3 %), *Paenibacillus barengoltzii* SAFN-016^T (93.3 %) and *Paenibacillus sanguinis* 2301083^T (93.2 %). Similar tree topologies were obtained in phylogenetic trees generated with the maximum-parsimony (see Supplementary Fig. S2 in IJSEM Online) and maximum-likelihood algorithms (data not shown).

DNA–DNA relatedness experiments were not performed between strain ZL^T and its closest phylogenetic neighbours as the level of 16S rRNA gene sequence similarity between strain ZL^T and other *Paenibacillus* species was less than 95 % and thus below the cut-off value (97 %) suggested by Stackebrandt & Goebel (1994) for genomic distinction of species.

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the European Molecular Biology Laboratory database (accession numbers are given in parentheses) constructed after multiple alignments of data showing the position of strain ZL^T within the genus *Paenibacillus*. Distances and clustering with the neighbour-joining method were performed by using the BioEdit software package. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. Bar, 1 % sequence dissimilarity per nucleotide position.

The DNA G+C content was determined in duplicate as described by Mesbah *et al.* (1989). The DNA G+C content of strain ZL^T was 49.2 (±1.2) mol% (mean±SD).

Chemotaxonomic studies were performed to establish whether strain ZL^T had a chemical profile consistent with its assignment within the genus *Paenibacillus*. Biomass for chemical studies was grown in shake flasks of trypticase soy broth for 5 days at 37 °C, checked for purity, harvested by centrifugation, washed twice in distilled water and freeze-dried. Polar lipids were extracted and analysed by two-dimensional TLC as described by Ventosa *et al.* (1993). The major cellular phospholipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and one unknown phosphoglycolipid. Respiratory quinones of strain ZL^T were extracted, separated and identified as described by Saha *et al.* (2005). The predominant isoprenologue was unsaturated menaquinone with seven isoprene units (MK-7). The diagnostic cell-wall amino acid of strain ZL^T was determined by TLC (Staneck & Roberts, 1974). The isolate contained *meso*-diaminopimelic acid (wall chemotype III). Cellular fatty acids were analysed as methyl esters by GC according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The predominant fatty acids of strain ZL^T were C_{16:0}, anteiso-C_{15:0} and iso-C_{16:0}. All of these properties are typical of representatives of the genus *Paenibacillus* (Shida *et al.*, 1997a). The fatty acid profile of strain ZL^T was generally comparable with those of the type strains of *P. assamensis*, *P. timonensis*, *P. macerans* and *P. sanguinis* (Shida *et al.*, 1997b; Roux & Raoult, 2004; Saha *et al.*, 2005), but differed in the proportions of certain fatty acids (Table 1). Strain ZL^T contained higher amounts of C_{16:0} (33.5%) and C_{14:0} (7.5%), a feature that enabled the novel strain to be clearly distinguished from *P. assamensis*, *P. timonensis*, *P. macerans* and *P. sanguinis*.

Strain ZL^T was examined for a broad range of phenotypic and biochemical properties by using the API 20E, API 20NE, API ZYM (bioMérieux) and Microlog GN2 (Biolog) systems according to the manufacturers' instructions. Additionally, the sensitivity of strain ZL^T to different antibiotics was checked by using antibiotic discs (Oxoid).

Detailed results of the biochemical characterization and antibiotic sensitivity tests are given in Table 2 and in the species description below. It is apparent from the results (Table 2) that strain ZL^T can be distinguished from representatives of its close phylogenetic relatives by using a combination of phenotypic attributes, especially ONPG hydrolysis, nitrate reduction, the Voges–Proskauer reaction, optimum temperature for growth, acid production from various sugars (such as glucose, mannitol, arabinose, inositol, rhamnose and sucrose) and enzyme activities (catalase, oxidase and gelatinase).

It is clear from the genotypic and phenotypic data presented that strain ZL^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus fonticola* sp. nov. is proposed.

Table 1. Whole-cell fatty acid contents (% of total fatty acids) of strain ZL^T and related species of the genus *Paenibacillus*

Taxa: 1, strain ZL^T; 2, *P. macerans* ATCC 8244^T; 3, *P. sanguinis* 2301083^T; 4, *P. timonensis* 2301032^T; 5, *P. assamensis* GPTSA 11^T. Data for *P. macerans*, *P. sanguinis*, *P. timonensis* and *P. assamensis* were obtained from Shida *et al.* (1997b), Roux & Raoult (2004) and Saha *et al.* (2005). No fatty acid data are available for *P. barengoltzii* SAFN-016^T (Osman *et al.*, 2006). Fatty acid values of <1% are not shown.

Fatty acid	1	2	3	4	5
C _{14:0}	7.5	3.7		1.3	1.8
C _{15:0}			2.8	2.0	
C _{16:0}	33.5	17.9	10.1	10.6	3.5
C _{17:0}					1.6
iso-C _{14:0}	3.6	7.9	1.7	8.2	4.7
iso-C _{15:0}	5.7	2.6	6.8	10.1	6.0
iso-C _{16:0}	9.3	16.4	13.4	15.5	11.6
iso-C _{17:0}	2.0		5.8	1.9	1.6
anteiso-C _{15:0}	32.5	36.1	44.2	43.4	48.4
anteiso-C _{17:0}	2.8	12.2	6.8	4.0	5.7
C _{16:1} ω11c	1.5				6.2
C _{16:1} ω7c alcohol					5.0
iso-C _{17:1} ω10c					1.2
Summed feature 1*					1.6

*Summed feature 1 comprises iso-C_{17:1} I and/or anteiso-C_{17:1}.

Description of *Paenibacillus fonticola* sp. nov.

Paenibacillus fonticola (fon.ti.co'la. L. masc. n. *fons fontis* a spring, fountain; L. suf. *-cola* from L. masc. or fem. n. *incola* an inhabitant of a place, a resident; N.L. n. *fonticola* an inhabitant of a fountain).

Cells are facultatively anaerobic, Gram-variable, sporulating, motile, rod-shaped, 0.8–1.0 µm in diameter and 2.0–12.4 µm in length. Grows at pH 7–10 and at 20–45 °C. Positive (API 20NE and 20E) for catalase, urease, aesculin hydrolysis, gelatin hydrolysis, β-galactosidase, OPNG test, gelatinase and acid production from arabinose. Negative for oxidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, gluconate, caprate, adipate, malate, citrate, phenylacetate, lysine decarboxylase and ornithine decarboxylase, citrate utilization, H₂S production, tryptophan deaminase, acetoin production and acid production from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose and amygdalin. In API ZYM enzyme reactions, positive for C4 esterase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and β-galactosidase, but negative for alkaline phosphatase, C8 lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase

Table 2. Phenotypic and biochemical characteristics that differentiate strain ZL^T from representatives of closely related species of the genus *Paenibacillus*

Taxa: 1, strain ZL^T; 2, *P. macerans* ATCC 8244^T; 3, *P. sanguinis* 2301083^T; 4, *P. timonensis* 2301032^T; 5, *P. assamensis* GPTSA 11^T; 6, *P. barengoltzii* SAFN-016^T. Data for *P. macerans*, *P. sanguinis*, *P. timonensis*, *P. assamensis*, *P. barengoltzii* are taken from Shida *et al.* (1997b), Roux & Raoult (2004), Saha *et al.* (2005) and Osman *et al.* (2006). +, Positive; –, negative; w, weak response; ND, no data.

Characteristic	1	2	3	4	5	6
Isolation source	Warm spring	ND	Human blood	Human blood	Warm spring	Spacecraft assembly facility
Catalase	+	+	–	+	+	+
Oxidase	–	+	–	+	+	+
ONPG hydrolysis	+	+	ND	ND	–	+
Growth at 50 °C	–	+	–	+	–	+
Growth at 44 °C	+	+	+	+	–	+
Anaerobic growth	+	+	+	+	–	–
Nitrate reduction	–	+	–	w	–	+
Voges–Proskauer test	–	+	–	–	–	+
Gelatinase	+	–	–	–	+	–
Acid production from:						
Amygdalin	–	ND	+	+	w	ND
Arabinose	+	+	+	+	–	–
Glucose	–	+	–	+	+	–
Inositol	–	ND	–	–	–	ND
Mannitol	–	+	+	–	–	–
Melibiose	–	ND	+	+	–	–
Rhamnose	–	+	–	–	–	–
Sucrose	–	+	+	–	w	–
DNA G + C content (mol%)	49.2	52.2	ND	ND	41.2	ND

and α -fucosidase. The following carbon sources are oxidized (positive in the Biolog GN2 system): dextrin, Tween 80, D-cellobiose, D-fructose, α -D-glucose, D-mannose, D-melibiose, sucrose and D-trehalose. The following substrates are not utilized as carbon sources (negative in the Biolog GN2 system): α -cyclodextrin, glycogen, Tween 40, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, L-fucose, D-galactose, gentiobiose, *myo*-inositol, α -D-lactose, lactulose, maltose, D-mannitol, methyl β -D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citrate, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconate, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketovaleric acid, α -ketobutyric acid, α -ketoglutaric acid, DL-lactate, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ -amino-butyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butane-diol, glycerol, DL- α -glycerol phosphate, glucose 1-phosphate

and glucose 6-phosphate. Sensitive to ampicillin (10 μ g), cefotaxime (30 μ g), chloramphenicol (30 μ g), gentamicin (10 μ g), kanamycin (30 μ g), nalidixic acid (30 μ g), novobiocin (30 μ g), penicillin G (10 U), rifampicin (5 μ g), streptomycin (10 μ g) and tetracycline (30 μ g). The major fatty acids are C_{16:0}, anteiso-C_{15:0} and iso-C_{16:0}. The G + C content of the DNA is 49.2 mol%.

The type strain, ZL^T (= BCRC 17579^T = LMG 23577^T), was isolated from a water sample collected from Jhonglun warm spring, Chiayi County, Taiwan.

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