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Paenibacillus limicola sp. nov., isolated from tidal flat sediment

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Abstract

An aerobic, Gram-staining-variable, rod-shaped, endospore-forming and motile bacterial strain, designated CJ6^T, was isolated from a tidal flat on Ganghwa Island, South Korea. The isolate was characterized based on a polyphasic taxonomy approach. Strain CJ6^T grew optimally on R2A agar media at 30 °C and pH 7. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain CJ6^T belonged to the genus *Paenibacillus*, displaying the highest sequence similarity to *Paenibacillus vulneris* CCUG 53270^T (97.0 %) and clearly defined strain CJ6^T as a novel species within the genus. The G+C content of the genomic DNA was 49.9 mol%. The major polar lipid contents of strain CJ6^T were phosphatidylmonomethylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and unidentified glycolipids. MK-7 was detected as the major respiratory quinone. The dominant fatty acid was anteiso-$C_{15:0}$. Analyses of phylogenetic, phenotypic, biochemical and chemotaxonomic characteristics indicated that strain CJ6^T was distinguishable from its closely related type strains. Therefore, strain CJ6^T represents a novel species in the genus *Paenibacillus*, for which name *Paenibacillus limicola* sp. nov. is proposed; the type strain is CJ6^T (=KACC 19303^T=JCM 32079^T).

The genus *Paenibacillus* was first proposed by Ash *et al.* [1] as a member of the 'group 3 bacilli' within the genus *Bacillus* with 11 *Bacillus* species. At the time of writing, the genus contains more than 213 novel species with validly published names (www.bacterio.net/paenibacillus.html). Typically, members of this genus are either facultatively anaerobic or strictly aerobic, rod-shaped, comprise anteiso-$C_{15:0}$ as the major fatty acid, and show a range of the G+C content from 39 to 54 mol% [2]. The purpose of the present study was to establish the taxonomic position of the novel *Paenibacillus*-like strain CJ6^T based on polyphasic taxonomy.

Strain CJ6^T was isolated from the tidal flat of Ganghwa Island (37° 36′ 58.40″ N 126° 22′ 37.40″ E), South Korea through a standard dilution plating technique on R2A agar (BD) media. The strain was routinely cultivated on R2A at 30 °C for 3 days and preserved at −80 °C with 30 % (w/v) glycerol solution.

The amplification of the 16S rRNA gene was conducted using AccuPower PCR Premix (Bioneer) and the universal bacterial primers 27F and 1492R [3]. The PCR product was purified and sequenced by the automated DNA analyser (PRISM 3730XL; Applied Biosystems) at Solgent (Daejeon, Republic of Korea) using the sequencing primers 27F, 1492R, 785F and 805R [3, 4]. An almost-complete 16S rRNA sequence (1434 bp) was obtained by editing the sequence using SEQMAN PRO software. The sequence was aligned with those of related type strains obtained from the EzBioCloud database (www.ezbiocloud.net/) [5] using CLUSTAL_X [6]. Phylogenetic analysis was carried out by neighbour-joining [7] and maximum-likelihood methods [8] using the phangorn module [9] of the statistical package R. Bootstrap analysis was performed based on 1000 replicates for the evaluation of tree topology. A general time reverse model was used to build the maximum-likelihood topology after examining the best suited model by using PHYML 3.0.1 software [10]. Strain CJ6^T showed the highest 16S rRNA gene sequence similarity to *Paenibacillus vulneris* CCUG 53270^T (97.0 %). Phylogenetic analysis revealed that strain CJ6^T formed a distinct phyletic line in the clade with *P. vulneris* CCUG 53270^T, *Paenibacillus yunnanensis* YN2^T, *Paenibacillus periandrae* PM10^T and *Paenibacillus rigui* WPCB173^T (Fig. 1). The chromosomal DNA of the isolate was extracted by using the FastDNA SPIN Kit (MP Biomedicals). The DNA G+C content was determined by high-performance liquid chromatography (HPLC) according to the protocol developed by Mesbah *et al.* [11] and the results revealed that the genomic DNA G+C mol% of strain CJ6^T was 49.9.

The pure culture of strain CJ6^T was grown on five different standard bacteriological media including R2A agar, tryptic soy agar (TSA, BD), nutrient agar (NA, Conda), Luria–Bertani agar (LA, BD), and marine agar (MA, BD) to determine the suitable media to grow for further experiments. Growth was examined at different temperatures (4, 10, 15, 20, 25, 30,

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The GenBank accession number for the 16S rRNA gene sequence of the strain CJ6^T is KY056224.
Two supplementary figures are available with the online version of this article.

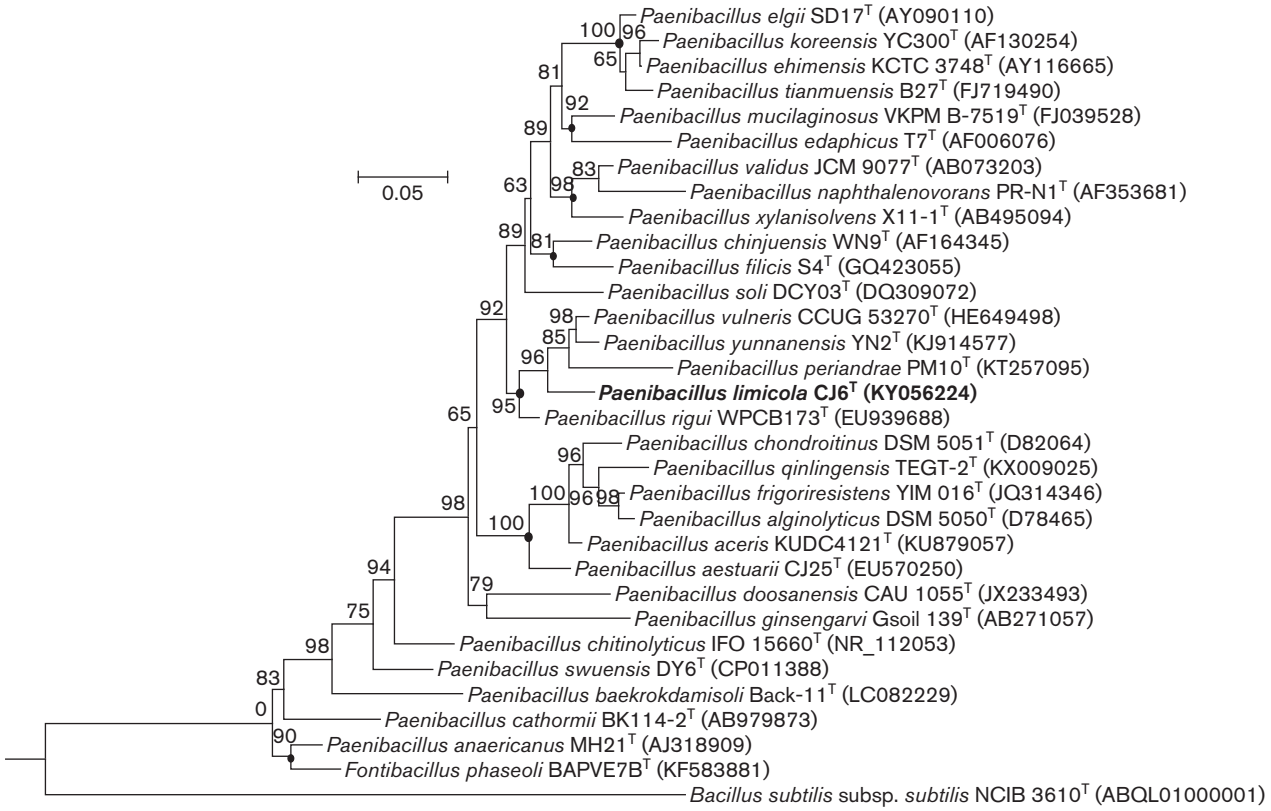

Fig. 1. Maximum-likelihood phylogenetic tree of strain CJ6^T and the type strains of related taxa based on an almost-complete 16S rRNA gene sequence. The tree was reconstructed using the neighbour-joining and maximum-likelihood algorithms. The numbers at nodes represent bootstrap values in percent (based on 1000 replicates) and only those more than 50 % are shown. Solid circles indicate generic branches that were present in both neighbour-joining and maximum-likelihood algorithms. *Bacillus subtilis* subsp. *subtilis* was used as an outgroup. Bar, 0.05 nucleotide substitutions per position.

37 and 40 °C) and at pH 4–11 (with 1.0 pH unit intervals) for 7 days under aerobic conditions. Salt tolerance was tested in R2A broth supplemented with 0–4 % (with 1 % intervals, w/v) NaCl at 30 °C for 7 days. Growth in an anaerobic state was determined using the GasPak EZ Pouch System (BD) at 30 °C for 15 days. The Gram reaction was analysed by following the manufacturer's protocol of a Gram staining kit (Sigma-Aldrich) and Ryu's non-staining KOH (3 % aqueous, w/v) test method [12]. The shape and size of the 3-day-old cells were observed by transmission electron microscopy (JEM 1010; JEOL). Motility was tested via observation of the 'brush or feather-like' growth in the semi-solid R2A (0.4 % agar, w/v) media. Spore formation was examined by staining with malachite green [13]. Oxidase and catalase activities were evaluated by the colour changing reaction of 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), and the O_2 bubble production in 3 % (v/v) H_2O_2 solution, respectively. Hydrolysis of casein (3 % skim-milk, w/v), cellulose (0.2 % sodium carboxymethyl cellulose, w/v), starch (1 %, w/v) and DNase (BD) was tested. Acid production from carbohydrates and enzymatic activities of the strain were determined using the API 20NE, API 50CH and API ZYM kits (bioMérieux) according to the manufacturer's protocols.

Except for MA, strain CJ6^T showed growth on R2A, TSA, NA and LA at temperatures from 15 to 37 °C (optimum 30 °C). It grew at pH 6–10 (optimum pH 7–8) in the presence of 0–1 % of NaCl (w/v). Colonies of strain CJ6^T was white-coloured, round, flat and smooth-edged on R2A agar, Gram-reaction-variable, KOH-negative, endospore-forming, and motile. The vegetative cells were rod-shaped, flagellated, 1.8–2.8 µm long and 0.5–0.8 µm wide (Fig. S1, available in the online version of this article). Strain CJ6^T showed positive reactions for oxidase and catalase activities. The isolate was able to hydrolyse starch and urea, but not casein, cellulose or DNA. Biochemical and physiological characteristics that differentiated strain CJ6^T from the reference strains are summarized in Table 1.

To analyse the chemotaxonomic characteristics, cells were cultivated on R2A agar at 30 °C for 48 h and harvested at an exponential growth stage. The lyophilized cells were used for the polar lipid and isoprenoid quinone analyses. Polar lipids were extracted with the aid of established protocols described previously [14, 15] on the basis of the two-dimensional silica gel thin-layer chromatography analysis. Specific groups of lipids were visualized using detection reagents:

Table 1. Differential physiological and biochemical characteristics of strain CJ6^T and its closely related type strains of the genus *Paenibacillus*

Strains: 1, CJ6^T; 2, *P. vulneris* CCUG 53270^T; 3, *P. rigui* WPCB173^T; 4, *P. periandrae* PM10^T. +, Positive; −, negative; v, variable; w, week. All data were obtained in this study.

Characteristic	1	2	3	4
Gram stain	v	+	v	v
Motility	+	–	+	+
Catalase	+	+	+	–
Growth at 40 °C	–	+	–	–
Growth in 2 % NaCl	–	+	+	w
Growth at pH 10	+	+	–	–
Hydrolysis of starch	+	–	–	–
Assimilation of:				
N-Acetylglucosamine	+	+	–	–
Arabinose	–	+	+	+
Mannose	–	–	+	+(−)*
Mannitol	–	+	+	− (+)*
Malate	–	+	+	+
Enzymatic activities of:				
Alkaline phosphatase	+	+	–	+
β-Glucosidase	–	+	w	+
α-Fucosidase	+	+	–	w
Trypsin	+	–	–	–
Esterase (C4)	w	–	+	w
Acid production from:				
Starch	+	–	+	–
Amygdalin	+	–	+	+
Glycogen	+	–	+	–
N-Acetylglucosamine	+	–	+	w
Methyl α-D-mannoside	+	–	+	w
D-Xylose	–	–	+	+(−)*
D-Maltose	+	–	+	+
D-Galactose	–	+	+	+
D-Fructose	–	+	+	+
D-Melezitose	–	–	+	–
D-Lyxose	–	+	–	+(−)*
Inositol	–	–	+	+

*Data taken from [19] that differed from our own data are shown in parentheses.

5 % molybdophosphoric acid for total lipid, molybdenum blue spray (Sigma) for phospholipid, ninhydrin reagent for aminolipid and α-naphthol for glycolipid. The major respiratory quinone was prepared by the modified method described by Minnikin *et al.* [16] and detected by HPLC using a C18 column (25 cm×4.6 mm, 5 µm) [17]. Fatty acid profiles of strain CJ6^T and reference strains were analysed by gas–liquid chromatography and compared using the standard FAME method by using the Microbial Identification System (MIDI) version 6.1 and the TSBA6 6.10 database.

The predominant polar lipids of strain CJ6^T were phosphatidylmonomethylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and several unidentified glycolipids. Moreover, some minor lipid contents were also found as follows: unidentified aminophospholipids, an unidentified lipid and an unidentified phospholipid (Fig. S2). Similar patterns of major polar lipids were found elsewhere for *P. vulneris*, *P. rigui* and *P. periandrae* [18, 19]. However, these minor lipids were distinctive from the other closely related strains. Menaquinone with seven isoprene units (MK-7) was detected as the major respiratory quinone. The major cellular fatty acid of strain CJ6^T was anteiso-$C_{15:0}$ (39.4 %), which is consistent with other members of the genus *Paenibacillus* [20]. The detailed fatty acid profiles of strain CJ6^T and related strains are given in Table 2.

As revealed by phylogenetic analysis, strain CJ6^T forms a notable phyletic line among the recognized species of the genus *Paenibacillus*. Moreover, the results from DNA G+C content, polar lipid, respiratory quinone and fatty acid analyses are consistent with the traits listed in the genus description by Shida *et al.* [2, 20]. The unique polar lipid profile and several physiological and biochemical characteristics, including optimal growth temperature and pH, carbon source assimilation, enzyme activities, and acid production, clearly distinguished strain CJ6^T from other validly named species. Therefore, strain CJ6^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus limicola* sp. nov. is proposed.

DESCRIPTION OF *PAENIBACILLUS LIMICOLA* SP. NOV.

Paenibacillus limicola (li.mi′co.la. L. n. *limus* mud; L. suff. -*cola* (from L. n. *incola*), dweller; N.L. n. *limicola*, mud-dweller).

Cells are aerobic, Gram-staining-variable, KOH test-negative, 1.8–2.8×0.5–0.8 µm long, rod-shaped, endospore-forming and motile. Colonies on R2A are white-coloured, round, flat and smooth-edged after 5 days of inoculation. Growth occurs at a temperature range of 15–37 °C (optimum 30 °C), in 0–1 % NaCl and in pH 6–10 (optimum pH 7–8). Oxidase- and catalase-positive. Hydrolyses soluble starch, gelatin and urea, but does not hydrolyse casein, cellulose and DNA. API 20NE test results are positive for 4-nitrophenyl-β-D-galactopyranoside. Assimilation of glucose, maltose and *N*-acetylglucosamine is observed. No assimilation of arabinose, mannose, mannitol and malate occurs. Indole is not produced, and nitrate is not reduced. Acid is produced from methyl α-D-mannoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, raffinose, starch, glycogen, gentiobiose, D-turanose, D-maltose and glycerol, but not from inositol, D-xylose, D-galactose, D-fructose, D-melezitose and D-lyxose. Alkaline phosphatase, leucine arylamidase, trypsin, α-galactosidase, β-galactosidase, α-glucosidase and α-fucosidase activities are positive, but lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase activities are negative.

Table 2. Cellular fatty acid profiles (%) of CJ6^T and the related type species of the genus *Paenibacillus*

Strains: 1, CJ6^T; 2, *P. vulneris* CCUG 53270^T; 3, *P. rigui* WPCB173^T; 4, *P. periandrae* PM10^T. TR, Trace (<1.0 %); –, not detected. All data were obtained in this study.

Fatty acid	1	2	3	4
Straight-chain saturated:				
$C_{10:0}$	3.6	2.6	TR	TR
$C_{12:0}$	5.2	2.9	TR	TR
$C_{13:0}$	2.4	1.7	TR	–
$C_{14:0}$	2	1.5	TR	1.7
$C_{16:0}$	6.8	7.2	4.7	5.5
Hydroxy:				
$C_{11:0}$ 2OH	1.6	–	–	–
$C_{11:0}$ 3OH	1.2	–	–	–
iso-$C_{11:0}$ 3OH	2.0	1.4	–	–
Branched iso-saturated:				
$C_{10:0}$	1.2	TR	TR	–
$C_{12:0}$	1.1	TR	–	–
$C_{13:0}$	TR	TR	–	–
$C_{14:0}$	3.6	2.9	2.9	4
$C_{15:0}$	5.4	3.8	2.4	5.5
$C_{16:0}$	6.4	5.1	13.1	3.7
$C_{17:0}$	2.2	1.6	TR	TR
Branched anteiso-saturated:				
$C_{15:0}$	39.7	55.4	62.5	75.5
$C_{17:0}$	2.9	4.2	7.3	2.0
$C_{19:0}$	–	–	TR	–
Unsaturated:				
$C_{16:1}\omega 11c$	TR	TR	1.0	–
cyclo-$C_{19:0}\omega 8c$	1.1	TR	–	–
iso-$C_{15:1}\omega 9c$	1.4	–	–	–
iso-$C_{17:1}\omega 5c$	1.6	1.2	–	TR
Summed feature 7	1.3	TR	–	–

*Summed features represent groups of two fatty acids that could not be separated by gas–liquid chromatography with the MIDI system. Summed feature 7 comprises $C_{19:1}\omega 6c$ and/or $C_{19:1}\omega 7c$.

Esterase (C4), lipase esterase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are weak. The major polar lipids of strain CJ6^T are phosphatidylmonomethylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The predominant respiratory quinone is MK-7. The major cellular fatty acid is anteiso-$C_{15:0}$.

The type strain CJ6^T (=KACC 19303^T=JCM 32079^T) was isolated from the tidal flat of Ganghwa Island, South Korea. The genomic DNA G+C content of the type strain is 49.9 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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