Bacillus pallidus sp. nov., isolated from forest soil

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A Gram-positive bacterium, designated strain CW 7^T, was isolated from forest soil in Anhui Province, south-east China. Cells were strictly aerobic, motile with peritrichous flagella and rod-shaped. The strain grew optimally at 30–37 °C and pH 7.0–8.0. The major fatty acids of strain CW 7^T were anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0}. The predominant menaquinone was MK-7. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. The G + C content of the genomic DNA was 42.3 mol%. Phylogenetic analysis indicated that strain CW 7^T belonged to a monophyletic cluster within the genus *Bacillus* and showed 16S rRNA gene sequence similarities of less than 96.5 % to recognized species of the genus *Bacillus*. The results of the polyphasic taxonomic study, including phenotypic, chemotaxonomic and phylogenetic analyses, showed that strain CW 7^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus pallidus* sp. nov. is proposed. The type strain is CW 7^T (=KCTC 13200^T=CCTCC AB 207188^T=LMG 24451^T).

The genus *Bacillus* is characterized as containing Gram-positive or Gram-variable, aerobic, flagellated, endospore-producing, rod-shaped bacteria. Chemotaxonomically, members of the genus contain MK-7 as the predominant menaquinone, and iso- and anteiso-type branched 15:0 and 17:0 components as the major fatty acids (Ahmed *et al.*, 2007; Yoon *et al.*, 2003). During the course of an investigation of the bacterial community of a forest soil from Anhui Province, China, a bacterial strain, designated CW 7^T, was isolated and submitted to a polyphasic taxonomic study. We propose here that this strain represents a novel species of the genus *Bacillus*.

Strain CW 7^T was isolated and preserved according to the methods and by using the cultural media described by Zhou *et al.* (2007). To investigate its morphological, biochemical and physiological characteristics, strain CW 7^T was routinely cultivated by using TYB medium (0.3 % yeast extract, 0.2 % beef extract, 0.6 % tryptone, 0.3 % NaCl, 0.001 % FeCl₃ and, where necessary, 1.5 % agar; w/v) at

37 °C. Unless indicated otherwise, phenotypic characteristics were studied by using standard procedures (Smibert & Krieg, 1994) and those described by Zhou *et al.* (2007) and all experiments were conducted in triplicate. The morphological characteristics of strain CW 7^T were observed by using light microscopy (model XTL-3400; Olympus) and transmission electron microscopy (model H-7650; Hitachi) after incubation for 12–48 h at 37 °C on TYB agar. Preparation for electron microscopy was performed as described by Yoon *et al.* (2003). For the various physiological tests, API 20NE and API CHB test strips (bioMérieux) were applied according to the manufacturer's instructions.

PCR amplification of the 16S rRNA gene was performed as described by Li *et al.* (2007). The 16S rRNA gene sequence was aligned manually with reference sequences retrieved from the GenBank database following BLAST searches. A phylogenetic tree was constructed by using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining and maximum-parsimony methods. Bootstrap analysis was used to evaluate the tree topology of the clustering data by performing 1000 resamplings (Felsenstein, 1985).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CW 7^T is EU364818.

A maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain CW 7^T, and a two-dimensional thin-layer chromatograph of a polar lipid extract of strain CW 7^T are available as supplementary material with the online version of this paper.

Fig. 1. Transmission electron micrograph of a single cell of strain CW 7^T grown on TYB agar (pH 7.0) for 12 h at 37 °C. Bar, 2.0 μm.

Genomic DNA was prepared for the determination of the base composition following the procedure of Marmur (1961). The DNA G+C content was determined by reversed-phase HPLC (Supelcosil LC-18-S, Supelco; Agilent 1100) according to Mesbah *et al.* (1989), using *Escherichia coli* DH5α as a standard. Menaquinones were extracted from lyophilized cells and the samples were purified and analysed by using HPLC according to the procedures reported by Hu *et al.* (2001). The cellular fatty acid composition was determined as described by Sasser (1990) by using the Microbial Identification System (MIDI, Inc.). The isomer type of the diamino acid of the cell-wall peptidoglycan was determined by TLC following the method of Komagata & Suzuki (1987). Polar lipids were extracted, examined by two-dimensional TLC and identified by using the procedures of Minnikin *et al.* (1984).

Strain CW 7^T showed good growth on TYB medium, but only weak growth was observed on Luria–Bertani agar (Oxoid) or on marine agar (Difco). Cells of strain CW 7^T were motile with peritrichous flagella and rod-shaped (0.7–

Table 1. Phenotypic characteristics that differentiate strain CW 7^T (*Bacillus pallidus* sp. nov.) from phylogenetically related *Bacillus* species

Taxa: 1, strain CW 7^T (data from this study); 2, *B. flexus* (Lim *et al.*, 2006; Heyrman *et al.*, 2005; Suresh *et al.*, 2004); 3, *B. simplex* (Lim *et al.*, 2006; Heyrman *et al.*, 2005); 4, *B. marisflavi* (Yoon *et al.*, 2003); 5, *B. aquimaris* (Yoon *et al.*, 2003); 6, *B. infantis* (Ko *et al.*, 2006); 7, *B. benzoeverans* (Pettersson *et al.*, 1996, 2000); 8, *B. circulans* (Pettersson *et al.*, 2000; Venkateswaran *et al.*, 2003); 9, *B. nealsonii* (Venkateswaran *et al.*, 2003); 10, *B. cohnii* (Suresh *et al.*, 2004); 11, *B. acidicola* (Albert *et al.*, 2005); 12, *B. subtilis* (Venkateswaran *et al.*, 2003; Lim *et al.*, 2006; Nakamura *et al.*, 1999). +, Positive; (+), weakly positive; –, negative; v, variable; NA, data not available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Flagellation	+	NA	+	+	+	NA	NA	NA	+	NA	+	+
Gram staining	+	NA	v	+ / v	v	+	+	+	+	NA	+	+
Catalase	(+)	NA	+	+	+	+	NA	NA	+	NA	+	+
Oxidase	–	NA	–	–	–	–	NA	–	–	NA	–	+
Colony colour*	LP	C	C	PY	POY	NA	NA	NA	B	W	Y	NA
Growth at 50 °C	–	–	–	–	–	NA	–	–	+	–	–	–
Optimal pH for growth	7.0–8.0	7.0	NA	6.0–8.0	6.0–7.0	NA	NA	NA	7.0	9.0	NA	NA
Growth in:												
5 % NaCl	–	+	v	+	+	NA	–	+	+	+	–	+
8 % NaCl	–	+	–	+	+	NA	–	NA	+	–	–	v
Hydrolysis of:												
Aesculin	+	–	v	+	–	+	NA	+	+	–	NA	+
Urea	–	–	–	–	–	NA	+	–	–	–	NA	–
Starch	–	+	+	–	+	+	–	+	+	NA	NA	+
Acid production from:												
D-Fructose	–	+	+	+	+	+	NA	+	+	NA	+	+
D-Glucose	–	+	+	+	+	+	–	+	+	–	+	+
Glycerol	–	+	+	+	–	–	NA	+	+	NA	+	+
Maltose	–	+	+	+	+	+	NA	+	+	–	+	+
D-Mannitol	–	+	+	+	–	+	–	+	+	–	+	+
Raffinose	–	+	–	(+)	–	+	NA	–	+	–	NA	+
Sucrose	–	+	+	+	+	+	NA	+	+	–	+	+
Trehalose	–	+	+	+	+	+	NA	+	+	NA	NA	+
D-Xylose	+	–	–	+	–	+	–	+	+	–	+	v

*B, Beige; C, cream; LP, light pink; POY, pale orange yellow; PY, pale yellow; W, white; Y, yellow.

1.0 µm in width and 1.8–3.5 µm in length) (Fig. 1). Those physiological and biochemical properties useful for the differentiation of strain CW 7^T from its closest phylogenetic neighbours are detailed in Table 1.

The almost-complete 16S rRNA gene sequence (1413 nt) of strain CW 7^T was determined. Neighbour-joining analysis of the 16S rRNA gene sequence showed that strain CW 7^T clearly belonged to the genus *Bacillus* and showed moderately low levels of sequence similarity (<96.5%) to the type strains of recognized *Bacillus* species. Strain CW 7^T showed levels of 16S rRNA gene sequence similarity of 96.1–95.5% to its closest phylogenetic neighbours (*Bacillus flexus* DSM 1320^T, *Bacillus simplex* DSM 1321^T and *Bacillus cohnii* DSM 6307^T), and these four strains formed a separate branch in the neighbour-joining phylogenetic tree (Fig. 2). The maximum-parsimony phylogenetic tree showed essentially the same position for strain CW 7^T as described above (see Supplementary Fig. S1, available in IJSEM Online).

The DNA G+C content of strain CW 7^T was 42.3 mol%. Its predominant menaquinone was MK-7. The polar lipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two ninhydrin-positive phosphoglycolipids and an unknown polar lipid

(see Supplementary Fig. S2 in IJSEM Online). The major fatty acids were iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}; the detailed fatty acid profiles of strain CW 7^T and its closest phylogenetic neighbours are given in Table 2. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid.

Although chemotaxonomic markers (menaquinone, major fatty acids, cell-wall peptidoglycan diaminopimelic acid and polar lipids) and phylogenetic analysis unequivocally supported that strain CW 7^T was a member of the genus *Bacillus*, it could be easily differentiated from other related *Bacillus* species by means of phenotypic properties such as morphological characteristics, acid production from carbohydrates (API CHB test strip; Table 1) and also by differences in minor fatty acid components (Table 2). On the basis of the phylogenetic and chemotaxonomic evidence together with the comparative phenotypic characteristics presented herein, strain CW 7^T is considered to represent a novel species of the genus *Bacillus*, for which the name *Bacillus pallidus* sp. nov. is proposed.

Description of *Bacillus pallidus* sp. nov.

Bacillus pallidus (pal.li'dus. L. masc. adj. *pallidus* pale, the light pink colour of colonies).

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain CW 7^T. Bootstrap values are shown as percentages of 1000 replicates; only values above 70% are shown. GenBank accession numbers are given in parentheses. Bar, 0.5 nucleotide substitutions per 100 nt.

Table 2. Cellular fatty acid composition of strain CW 7^T (*Bacillus pallidus* sp. nov.) and phylogenetically related *Bacillus* species

Taxa: 1, strain CW 7^T (data from this study); 2, *B. flexus* (Suresh *et al.*, 2004); 3, *B. simplex* (Heyrman *et al.*, 2005); 4, *B. marisflavi* (Yoon *et al.*, 2003); 5, *B. aquimaris* (Yoon *et al.*, 2003); 6, *B. infantis* (Ko *et al.*, 2006); 7, *B. circulans* (Venkateswaran *et al.*, 2003); 8, *B. nealsonii* (Venkateswaran *et al.*, 2003); 9, *B. cohnii* (Suresh *et al.*, 2004); 10, *B. acidicola* (Albert *et al.*, 2005); 11, *B. subtilis* (Venkateswaran *et al.*, 2003). Values are percentages of total fatty acids. ND, Not detected. Fatty acids representing less than 1.0 % in all species were omitted. Means $\pm$ SD are given where more than one test was performed.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
iso-C _{14:0}	1.2 $\pm$ 0.3	1.6	10.8 $\pm$ 4.3	9.1	6.5	1.9	4.0	6.6	0.8	ND	2.3
iso-C _{14:0} 3-OH	ND	ND	ND	2.0	ND	ND	ND	ND	ND	ND	ND
C _{14:0}	3.4 $\pm$ 0.8	ND	2.8 $\pm$ 0.2	1.7	0.9	1.5	2.9	12.3	ND	0.91	1.1
iso-C _{15:0}	11.5 $\pm$ 1.3	32.2	13.5 $\pm$ 1.2	22.9	46.9	44.0	13.9	26.4	22.9	57.9	13.2
iso-C _{15:0} 3-OH	ND	ND	ND	6.1	ND	ND	ND	ND	ND	ND	ND
anteiso-C _{15:0}	36.6 $\pm$ 2.8	35.4	48.5 $\pm$ 4.1	27.7	22.2	30.9	58.4	32.2	20.3	24.3	40.0
C _{15:0}	ND	ND	<1.0	1.1	0.5	ND	1.0	ND	0.2	ND	ND
C _{15:0} 2-OH	ND	ND	ND	5.5	ND	ND	ND	ND	ND	ND	ND
iso-C _{16:0}	5.6 $\pm$ 1.1	8.6	4.9 $\pm$ 1.4	7.7	4.2	2.7	4.4	2.1	7.0	0.81	4.6
C _{16:0}	7.7 $\pm$ 0.9	1.2	5.4 $\pm$ 0.6	1.0	0.5	1.3	2.7	5.2	1.0	0.83	10.2
iso-C _{16:0} 3-OH	ND	ND	ND	1.5	ND	ND	ND	ND	ND	ND	ND
C _{16:1} ω 7c alcohol	ND	ND	3.3 $\pm$ 0.6	2.5	8.4	1.9	0.2	1.8	ND	ND	ND
C _{16:1} ω 11c	ND	ND	6.6 $\pm$ 0.7	ND	1.3	1.2	2.6	7.5	ND	0.7	4.8
iso-C _{17:0}	1.5 $\pm$ 0.1	2.1	1.0 $\pm$ 0.2	1.2	0.8	1.9	1.3	ND	35.3	3.3	7.8
iso-C _{17:1} ω 10c	ND	ND	ND	ND	1.8	1.0	0.1	ND	ND	1.7	1.6
anteiso-C _{17:0}	19.0 $\pm$ 3.1	3.4	1.9 $\pm$ 0.5	7.1	2.3	7.4	4.8	3.0	2.2	8.5	12.2
C _{17:0} 2-OH	ND	ND	ND	1.2	ND	ND	ND	ND	ND	ND	ND
C _{18:0}	4.1 $\pm$ 1.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.0
C _{18:1} ω 9c	3.4 $\pm$ 0.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Summed features*											
4	ND	ND	ND	1.8	2.9	3.1	0.3	ND	ND	2.0	1.2
8	1.8 $\pm$ 0.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Summed features represent groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed feature 4 contains iso-C_{17:1} I and/or anteiso-C_{17:1} B; summed feature 8 contains C_{18:1} ω 7c and/or C_{18:1} ω 6c.

Cells are Gram-positive, motile by means of peritrichous flagella, spore-forming and rod-shaped (0.7–1.0 $\times$ 1.8–3.5 μ m). Colonies are circular, flat and light pink after 2 days cultivation at 37 °C on TYB medium. Growth occurs at 15–42 °C (optimum 30–37 °C) and pH 6.0–8.5 (optimum pH 7.0–8.0). Grows in the absence of NaCl but does not grow in the presence of 3 % NaCl. Positive for hydrolysis of casein and gelatin, but negative for hydrolysis of Tween 80, chitin, starch, DNA, pectin and tyrosine. Weak catalase activity is present. Oxidase, β -galactosidase, arginine dihydrolase, lipase, methyl α -D-glucosidase, ornithine decarboxylase, lysine decarboxylase and urease activities are absent. Nitrate and nitrite are not reduced. H₂S (triple-sugar iron test) is not produced. Voges–Proskauer test is positive. Indole is not produced. Utilizes aesculin, gluconate, 2-ketogluconate and D-xylose, but not N-acetylglucosamine, D-adonitol, D-arabinose, L-arabinose,

D-arabitol, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D-fructose, D-fucose, L-fucose, D-galactose, D-gentio-biose, D-glucose, glycerol, glycogen, inositol, inulin, 5-ketogluconate, D-lactose, amygdalin, D-lyxose, maltose, mannitol, D-mannose, melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, methyl β -D-xyloside, raffinose, L-rhamnose, D-ribose, D-salicin, sorbitol, L-sorbitose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol or L-xylose (API CHB tests). Predominant isoprenoid quinone is MK-7. Major cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The DNA G+C content of the type strain is 42.3 mol% (HPLC).

The type strain, CW 7^T (=KCTC 13200^T=CCTCC AB 207188^T=LMG 24451^T), was isolated from a forest soil in Anhui Province, China.

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