Bacillus korlensis sp. nov., a moderately halotolerant bacterium isolated from a sand soil sample in China

Lei Zhang, Yang Wang, Jun Dai, Yali Tang, Qiao Yang, Xuesong Luo and Chengxiang Fang

Correspondence
Chengxiang Fang
cxfang@whu.edu.cn

College of Life Sciences, Wuhan University, Wuhan 430072, PR China

A Gram-positive-staining, rod-shaped, motile, spore-forming and moderately halotolerant bacterium, designated ZLC-26^T, was isolated from a sand soil sample collected from Xinjiang Province, China, and was characterized by using a polyphasic taxonomic approach. This isolate grew optimally at 30–37 °C and pH 7–8. It grew with 0–8% NaCl (optimum, 0–2%). Comparative 16S rRNA gene sequence analysis showed that strain ZLC-26^T was closely related to members of the genus *Bacillus*, exhibiting the highest 16S rRNA gene sequence similarities to *Bacillus nealsonii* DSM 15077^T (97.1%), *B. shackletonii* LMG 18435^T (97.0%), *B. siralis* 171544^T (97.0%), *B. circulans* IAM 12462^T (96.7%) and *B. pocheonensis* Gsoil 420^T (96.7%). Strain ZLC-26^T contained MK-7 as the predominant menaquinone. The diagnostic diamino acid in the cell-wall peptidoglycan was *meso*-diaminopimelic acid. The major cellular fatty acids were iso-C_{15:0}, C_{16:1ω11c} and anteiso-C_{15:0}. The DNA G+C content was 38.2 mol%. These chemotaxonomic results supported the affiliation of strain ZLC-26^T to the genus *Bacillus*. However, low DNA–DNA relatedness values and distinguishing phenotypic characteristics allowed genotypic and phenotypic differentiation of strain ZLC-26^T from recognized *Bacillus* species. On the basis of the evidence presented, strain ZLC-26^T is considered to represent a novel species of the genus *Bacillus*, for which the name *Bacillus korlensis* sp. nov. is proposed. The type strain is ZLC-26^T (=CCTCC AB 207172^T=NRRL B-51302^T).

Since the genus *Bacillus* was first described by Cohn in 1872, the number of *Bacillus* species has fluctuated widely. Currently, many species originally described as *Bacillus* have been transferred to related genera. Nevertheless, the genus *Bacillus* is still considered to be one of the largest genera and currently includes more than 200 species (Euzéby, 2008). Novel *Bacillus* species from diverse habitats have been described recently (Aino *et al.*, 2008; Albuquerque *et al.*, 2008; Kuisiene *et al.*, 2008). During the course of an investigation of the culturable bacterial community in soil from a sand dune area in Xinjiang Province, China, a large number of bacteria were isolated. In this study, we report on the taxonomic characterization of one of these isolates, designated strain ZLC-26^T. On the basis of phenotypic characteristics, chemotaxonomic data,

phylogenetic analysis and DNA–DNA hybridization, the isolate represents a novel species of the genus *Bacillus*.

Strain ZLC-26^T was isolated from a sand soil sample collected from Xinjiang Province, China. The soil sample was thoroughly suspended in sterilized water and serial dilutions of the suspension were spread on marine agar 2216 (MA; Difco). Isolation was achieved after incubation at 30 °C for 1 week. The isolate ZLC-26^T was routinely cultivated on the same medium at 30 °C and maintained as a glycerol suspension (20%, v/v) at –80 °C. The type strains of seven *Bacillus* species were used as reference strains; *Bacillus nealsonii* DSM 15077^T, *B. shackletonii* DSM 18868^T, *B. siralis* DSM 13140^T, *B. pocheonensis* DSM 18135^T and *B. benzoovorans* DSM 5391^T were obtained from the DSMZ, and *B. circulans* IAM 12462^T and *B. firmus* IAM 12464^T were obtained from the China Center for Type Culture Collection (CCTCC), China.

Cell morphology, size and motility were examined by using phase-contrast microscopy (BX51 microscope; Olympus). The Gram reaction was carried out according to the classic Gram procedure described by Doetsch (1981). Flagella were observed from silver-nitrate-stained cells of strain

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZLC-26^T is EU603328.

Maximum-parsimony and maximum-likelihood trees based on 16S rRNA gene sequences showing the phylogenetic relationship of strain ZLC-26^T with related taxa are available as supplementary material with the online version of this paper.

ZLC-26^T that were incubated on MA slants at 30 °C for 12 h (Rhodes, 1958). Growth at different temperatures (4, 7, 10, 17, 25, 30, 37, 42, 45 and 50 °C) was investigated on MA for up to 1 week. Growth at various NaCl concentrations (0–10 %, w/v, with increments of 1 %) was investigated in trypticase soy broth (Difco) prepared according to the formula except that NaCl was excluded. The pH range for growth was determined in marine broth 2216 (MB; Difco) at pH 4.0–11.0, by using the following buffer system: pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH₂PO₄/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO₃/0.1 M Na₂CO₃; pH 11.0, 0.05 M Na₂HPO₄/0.1 M NaOH. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber (AnaeroJar; Oxoid) for 1 week on MA at 30 °C. Oxidase activity was determined using a 1 % solution of tetramethyl *p*-phenylenediamine (Kovács, 1956). Catalase activity was detected by assessing the production of bubbles after the addition of a drop of 3 % H₂O₂. Hydrolysis of starch and casein was tested as described by Smibert & Krieg (1994) on MA. Tests were also made for hydrolysis of CM-cellulose (0.1 %, w/v), chitin from crab shells (1 %, w/v) and tyrosine (0.5 %, w/v). Activities of constitutive enzymes and other physiological properties were determined by using the API 20E, API 20NE, API 50 CH (with API 50 CHB/E medium) and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Antimicrobial susceptibility testing was performed by the agar-diffusion method using antibiotic-impregnated discs (Oxoid) as described by Buczolits *et al.* (2002).

For 16S rRNA gene sequencing and phylogenetic analysis, DNA was extracted using a commercial genomic DNA extraction kit (ChaoShi-Bio; China). The primer pair 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1527r (5'-AGAAAGGAGGTGATCCAGCC-3') was used for amplification of the 16S rRNA gene (Lane, 1991). PCR and 16S rRNA gene sequencing were carried out as described by Lin *et al.* (2004). The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic analysis was performed by using MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the data via CLUSTALX (Thompson *et al.*, 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering by neighbour-joining, and a discrete character-based maximum-parsimony method were used. The relationships among taxa were also established with the maximum-likelihood algorithm in the PHYLIP 3.66 package (Felsenstein, 2006). In each case, bootstrap values were calculated based on 1000 replications.

For DNA–DNA hybridization experiments and determination of the DNA G + C content, genomic DNA of strain ZLC-26^T and four reference strains was prepared according to a modification of the procedure of Wilson (1987). The DNA G + C content of strain ZLC-26^T was determined by

HPLC (UltiMate 3000; Dionex) according to the method of Mesbah *et al.* (1989). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values. The respiratory quinone system was extracted and determined by HPLC as described by Xie & Yokota (2003). The isomer type of diaminopimelic acid in the peptidoglycan was determined by using the method described by Stanek & Roberts (1974). For the determination of the cellular fatty acid methyl ester content, strain ZLC-26^T and five of the reference strains (except *B. pocheonensis* DSM 18135^T and *B. benzoovorans* DSM 5391^T) were grown on trypticase soy agar (TSA; Difco) at 30 °C for 24 h. Because of poor growth on this medium, *B. pocheonensis* DSM 18135^T was grown on R2A agar (Difco) and *B. benzoovorans* DSM 5391^T was grown on nutrient agar (NA; Difco) supplemented with 0.4 % yeast extract and 0.2 % benzoate. Fatty acid methyl ester analysis was carried out according to the standard protocol of the Sherlock Microbial Identification System (MIDI, 1999).

Strain ZLC-26^T formed cream-yellow, circular, slightly raised colonies when grown on MA at 30 °C for 2 days. Cells were Gram-positive-staining, rod-shaped, spore-forming, strictly aerobic and motile by means of peritrichous flagella. Spores were mainly oval and lie in subterminal (occasionally paracentral) positions in slightly swollen sporangia. Other phenotypic features of strain ZLC-26^T are summarized in the species description and a comparison of selective characteristics with closely related type strains is given in Table 1.

For strain ZLC-26^T, 1471 bp of the 16S rRNA gene sequence was determined. Comparative 16S rRNA gene sequence analysis showed that strain ZLC-26^T was most closely related to members of the genus *Bacillus*. Strain ZLC-26^T exhibited the highest 16S rRNA gene sequence similarities to *B. nealsonii* DSM 15077^T (97.1 %), *B. shackletonii* LMG 18435^T (97.0 %), *B. siralis* 171544^T (97.0 %), *B. circulans* IAM 12462^T (96.7 %), *B. pocheonensis* Gsoil 420^T (96.7 %), *B. acidicola* 105-2^T (96.6 %), *B. isabelliae* CVS-8^T (96.5 %), *B. firmus* IAM 12464^T (96.5 %) and *B. benzoovorans* DSM 5391^T (96.5 %). The 16S rRNA gene sequence similarities to the type strains of all other *Bacillus* species with validly published names were below 96.4 %. In the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1), strain ZLC-26^T fell within a coherent cluster comprising *B. nealsonii* DSM 15077^T, *B. siralis* 171544^T, *B. circulans* IAM 12462^T, *B. benzoovorans* DSM 5391^T and *B. firmus* IAM 12464^T. The topologies of the phylogenetic trees generated using the maximum-parsimony and maximum-likelihood algorithms (Supplementary Figs S1 and S2, available in IJSEM Online) were somewhat different from that of the tree constructed using the neighbour-joining method.

Table 1. Differential phenotypic characteristics of strain ZLC-26^T and some related *Bacillus* species

Strains: 1, *Bacillus korlensis* sp. nov. ZLC-26^T (data from this study); 2, *B. nealsonii* DSM 15077^T (Venkateswaran *et al.*, 2003); 3, *B. shackletonii* DSM 18868^T (Logan *et al.*, 2004; Albuquerque *et al.*, 2008); 4, *B. siralis* DSM 13140^T (Pettersson *et al.*, 2000); 5, *B. circulans* IAM 12462^T (Claus & Berkeley, 1986; Pettersson *et al.*, 2000); 6, *B. pocheonensis* DSM 18135^T (Ten *et al.*, 2007); 7, *B. firmus* IAM 12464^T (Claus & Berkeley, 1986; Pettersson *et al.*, 2000); 8, *B. benzoovorans* DSM 5391^T (Pichinoty *et al.*, 1984; Pettersson *et al.*, 2000); 9, *B. acidicola* 105-2^T (Albert *et al.*, 2005; Albuquerque *et al.*, 2008); 10, *B. isabelliae* CVS-8^T (Albuquerque *et al.*, 2008). For acid production, data for columns 1–8 are from this study and for columns 9–10 from Albuquerque *et al.* (2008), all with API 50 CH strips. o, Oval; e, ellipsoidal; +, positive; –, negative; (+), weakly positive; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Spore shape	o	o	e	o	o	e	o	o	e	ND
Swollen sporangia	+	–	+	+	+	+	–	–	+	+
Anaerobic growth	–	+	–	–	+	–	–	–	–	–
Growth conditions										
NaCl 5 %	+	+	–	+	+	+	+	–	–	+
NaCl 10 %	–	–	–	–	–	+	+	–	–	+
50 °C	–	+	+	+	–	–	–	–	–	–
Nitrate reduction	+	–*	–	+	v	+	+	+	–	–
Hydrolysis of:										
Aesculin	+	+	+	+	+	+	–	–*	ND	+
Casein	–	+	v	+	–	–	+	+	–	–
Gelatin	–	–*	v	+	+	–	+	–	–	–
Starch	+	+	–	–	+	–	+	–	+	+
Acid production from:										
L-Arabinose	+	–	–	–	+	(+)	–	–	ND	–
D-Galactose	+	–	(+)	–	+	(+)	–	–	+	–
D-Glucose	+	+	+	+	+	+	+	–	+	–
Glycerol	(+)	–	–	+	(+)	(+)	+	–	+	–
Glycogen	+	–	–	–	+	–	–	–	ND	+
Lactose	+	+	(+)	–	+	–	–	–	+	–
D-Lyxose	–	+	–	–	–	–	–	–	ND	–
D-Mannose	–	(+)	(+)	+	+	+	–	–	+	–
L-Rhamnose	+	+	–	–	–	+	–	–	ND	–
D-Ribose	+	–	(+)	–	–	–	–	–	+	(+)
D-Tagatose	–	(+)	(+)	–	–	–	–	–	ND	+
Sucrose	+	+	–	–	+	+	+	–	+	(+)
D-Xylose	+	(+)	–	–	+	(+)	–	–	+	(+)
L-Arabitol	–	–	–	–	(+)	–	–	–	ND	–
D-Sorbitol	–	+	–	–	+	–	–	–	ND	–
Xylitol	–	–	–	–	+	–	–	–	ND	–
Methyl α -D-mannopyranoside	–	–	–	–	+	–	–	–	ND	–
Methyl β -D-xylopyranoside	–	(+)	–	–	(+)	–	–	–	ND	–
DNA G+C content (mol%)	38.2	ND	35.4	ND	35.7	44.9	41.4	41.3 $\pm$ 1.1	43.2	37.9

*Data from this study.

Nevertheless, strain ZLC-26^T formed a branch with *B. siralis* 171544^T in these three phylogenetic trees. The DNA G+C content of strain ZLC-26^T was 38.2 mol%, which differs slightly from its phylogenetically closest relatives (Table 1).

To differentiate strain ZLC-26^T from its closely related species, DNA–DNA hybridization was performed. Levels of DNA–DNA relatedness between strain ZLC-26^T and *B. nealsonii* DSM 15077^T, *B. shackletonii* DSM 18868^T, *B. siralis* DSM 13140^T and *B. circulans* IAM 12462^T were 21, 11, 17 and 35 %, respectively. These values indicate that

strain ZLC-26^T represents a genomic species that is distinct from these four *Bacillus* species (Wayne *et al.*, 1987).

The diagnostic diamino acid in the cell-wall peptidoglycan of strain ZLC-26^T was *meso*-diaminopimelic acid. The predominant isoprenoid quinone was MK-7. These characteristics are in agreement with those of numerous species of the genus *Bacillus* (Claus & Berkeley, 1986). The major fatty acids of strain ZLC-26^T were iso-C_{15:0} (35.2 %), C_{16:1 ω 11c} (16.1 %) and anteiso-C_{15:0} (15.6 %). As shown in Table 2, some qualitative and quantitative differences in fatty acid contents could be observed

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain ZLC-26^T and related taxa. Percentage bootstrap values based on 1000 replications are given at branch points. *Brevibacillus brevis* JCM 2503^T (D78457) was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

between strain ZLC-26^T and its closely related species. In particular, in comparison with *B. nealsonii* DSM 15077^T, *B. shackletonii* DSM 18868^T, *B. siralis* DSM 13140^T, *B. circulans* IAM 12462^T and *B. firmus* IAM 12464^T, strain ZLC-26^T contained larger amounts of C_{16:1}ω11c and summed feature 3. Additionally, strain ZLC-26^T could be differentiated from *B. siralis* DSM 13140^T by the significantly smaller amount of iso-C_{17:1}ω10c.

Therefore, on the basis of the data presented, strain ZLC-26^T should be placed in the genus *Bacillus* as representing a novel species, for which the name *Bacillus korlensis* sp. nov. is proposed.

Description of *Bacillus korlensis* sp. nov.

Bacillus korlensis (kor.len'sis. N.L. masc. adj. *korlensis* pertaining to Korla, a city of Xinjiang Province in the north-west of China from where the type strain was isolated).

Cells are Gram-positive-staining, rod-shaped, strictly aerobic, spore-forming, 1.8–3.0 μm long and 0.7–0.8 μm wide. Cells are motile by means of peritrichous flagella. Endospores are oval, lie subterminally, occasionally paracentrally, and usually cause the sporangia to swell. Growth occurs at 7–45 °C; optimal temperature is 30–37 °C. Growth occurs at pH 6.0–11.0, with optimal growth at pH 7.0–8.0. Growth occurs at 0–8 % (w/v) NaCl (optimum, 0–2 %). Oxidase- and catalase-positive. Nitrate is reduced to nitrite. Hydrolyses aesculin and

starch. Does not hydrolyse casein, gelatin, cellulose, chitin or tyrosine. H₂S production, citrate utilization, indole production and urease are negative. Acid is produced from glycerol (weakly), L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, L-rhamnose, inositol, D-mannitol, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose (weakly), starch, glycogen, gentiobiose, turanose and potassium gluconate (weakly), but not from erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-mannose, L-sorbose, dulcitol, D-sorbitol, methyl α-D-mannopyranoside, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate. According to the API ZYM gallery, produces alkaline phosphatase, esterase (C4), esterase lipase (C8), α-chymotrypsin (weakly), acid phosphatase (weakly), naphthol-AS-BI-phosphohydrolase, β-glucuronidase (weakly), α-glucosidase and β-glucosidase, but not lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Susceptible to chloramphenicol, kanamycin, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline and vancomycin. The cell-wall peptidoglycan contains meso-diaminopimelic acid. The predominant menaquinone is MK-7. Major fatty acids are iso-C_{15:0}, C_{16:1}ω11c and anteiso-C_{15:0}. The DNA G + C content of the type strain is 38.2 mol%.

Table 2. Cellular fatty acid compositions (%) of strain ZLC-26^T and some related *Bacillus* species

Strains: 1, *Bacillus korlensis* sp. nov. ZLC-26^T; 2, *B. nealsonii* DSM 15077^T; 3, *B. shackletonii* DSM 18868^T; 4, *B. sibiricus* DSM 13140^T; 5, *B. circulans* IAM 12462^T; 6, *B. firmus* IAM 12464^T; 7, *B. pocheonensis* DSM 18135^T; 8, *B. benzoovorans* DSM 5391^T. All data from this study. Data for columns 1–6 were obtained after growth on TSA at 30 °C for 24 h, for column 7 after growth on R2A agar at 30 °C for 24 h and for column 8 after growth on NA supplemented with 0.4 % yeast extract and 0.2 % benzoate at 30 °C for 24 h. –, Not detected.

Fatty acid	1	2	3	4	5	6	7	8
Straight-chain saturated								
C _{14:0}	3.0	7.8	1.6	0.7	2.5	1.8	3.1	3.2
C _{15:0}	–	–	–	0.4	–	–	2.2	0.5
C _{16:0}	4.7	3.5	0.4	0.8	2.3	1.4	3.6	17.6
C _{17:0}	0.1	0.1	0.1	0.1	–	–	0.3	0.3
C _{18:0}	0.4	0.2	–	–	0.1	0.6	–	0.8
Branched saturated								
iso-C _{13:0}	0.4	2.3	0.1	0.1	0.5	0.2	–	0.3
iso-C _{14:0}	3.7	9.5	0.5	1.2	5.5	2.7	18.3	14.9
iso-C _{15:0}	35.2	15.6	41.3	57.4	21.7	37.1	13.8	7.9
iso-C _{16:0}	0.8	3.5	2.9	1.2	6.4	2.5	15.3	5.2
iso-C _{17:0}	3.7	0.2	0.4	3.2	1.0	1.9	0.6	0.4
anteiso-C _{15:0}	15.6	38.7	23.7	10.4	47.3	16.8	28.1	28.4
anteiso-C _{17:0}	1.8	2.3	13.2	2.1	6.6	3.6	1.8	1.7
iso-C _{13:0} 3-OH	0.4	1.2	0.6	0.2	0.2	0.9	1.4	0.6
Mono-unsaturated								
C _{16:1} ω11c	16.1	5.2	1.8	2.2	1.2	3.1	1.1	7.4
C _{16:1} ω7c alcohol	3.2	1.5	0.5	3.1	1.7	5.3	1.8	0.7
iso-C _{17:1} ω10c	1.5	–	1.1	8.2	0.4	2.4	–	–
Summed features*								
3	4.1	0.3	0.1	–	–	0.4	–	–
4	1.4	0.2	3.3	2.1	0.6	11.4	–	–

*Summed features represent groups of two or three fatty acids that could not be separated by gas liquid chromatography with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 contained anteiso-C_{17:1} I. B and/or iso-C_{17:1} I.

The type strain, ZLC-26^T (=CCTCC AB 207172^T=NRRL B-51302^T), was isolated from a sand soil sample collected from Korla, Xinjiang Province, China.

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