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ORIGINAL PAPER

Bacillus salitolerans sp. nov., a novel bacterium isolated from a salt mine in Xinjiang province, China

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Abstract A novel aerobic bacterium, KC1^T, was isolated from a salt mine in Kuche county, Xinjiang province, China. Cells were observed to be Gram-positive, rod-shaped, endospore-forming and motile with flagella. Strain KC1^T was found to grow at 25–45 °C (optimum 37 °C), pH 6.5–9.0 (optimum 8.0) and NaCl 0–10 % (v/v) (optimum 4 %). The major fatty acids were identified as anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. Menaquinone-7 (MK-7) was found to be the predominant isoprenoid quinone. The cell-wall diamino acid was found to be *meso*-diaminopimelic acid. Polar lipid analysis revealed the presence of phosphatidylglycerol and a glycolipid. The 16S rRNA gene sequence of strain KC1^T showed low similarity (<96 %) to other validly named species. The phylogenetic trees showed that strain KC1^T is closely related to *Bacillus azotoformans* DSM 1046^T and *Bacillus methanolicus* DSM 16454^T. Both these type strains showed 95.4 % 16S rRNA gene sequence similarity to strain KC1^T. The DNA G+C content of strain KC1^T was determined to be 39.0 mol%. On the basis of its phenotypic, chemotaxonomic and genotypic characteristics, strain KC1^T is considered to represent a novel species of the genus *Bacillus*, for which the name *Bacillus salitolerans* sp. nov. is proposed. The type strain is KC1^T (=JCM 19760^T = CGMCC 1.12810^T).

Keywords *Bacillus salitolerans* sp. nov. · Polyphasic taxonomy · 16S rRNA gene · Salt mine

Introduction

The genus *Bacillus*, belonging to the family *Bacillaceae*, phylum *Firmicutes*, was firstly proposed by Cohn (1872). It is one of the largest bacterial genera, including around 200 validly named species and 7 subspecies (http://www.bacterio.net/bacillus.html). The species of this genus are phenotypically and genotypically heterogeneous (Priest 1993; Slepecky and Hemphill 2006). In general, *Bacillus* species are obligate aerobes or facultatively anaerobic, spore-forming, rod-shaped, Gram-positive, and have low G+C content (Claus and Berkeley 1986). Most members have MK-7 as the major respiratory quinone; iso-$C_{15:0}$ and anteiso-$C_{15:0}$ as the major fatty acids; and *meso*-diaminopimelic acid as the

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diagnostic diamino acid in the peptidoglycan. *Bacillus* species have been isolated from various habitats, including air (Shivaji et al. 2006), ancient tombs (Gatson et al. 2006), brown alga (Ivanova et al. 2004), desert sands (Zhang et al. 2011), forest soils (Chen et al. 2011), freshwater (Baik et al. 2010), ginseng root (Qiu et al. 2009), hot springs (Nazina et al. 2004), marine sediments (You et al. 2013; Zhu et al. 2014), sea water (Yoon et al. 2003), sandy soil (Lee et al. 2008) and a soda lake (Reddy et al. 2015). Many can be found in saline habitats (Amoozegar et al. 2009; Arahal et al. 1999; Lee et al. 2006; Lim et al. 2006; Pappa et al. 2010; Shi et al. 2011; Xue et al. 2008).

During our surveys of bacterial diversity of a salt mine in Kuche county, Akesu area in Xinjiang province of China, a novel isolate named KC1^T was obtained. Based on a polyphasic taxonomic approach, we propose that strain KC1^T represents a novel species of genus *Bacillus*.

Materials and methods

Bacterial strains and culture condition

Strain KC1^T was isolated from a salt mine sample (salt crystal, obtained at 41°43′0″N, 82°57′33″E) in Xinjiang province, China. Marine broth 2216 (MB, Difco) supplemented with 2.0 % of agar (MA) was used for bacterial isolation. For bacterial enrichment about 3 g of salt sample was suspended in 30 ml MB (28 °C, 2 days) which was further diluted and spread on MA plates. The plates were incubated at 28 °C for 2 days. A cream colony was isolated, purified and identified. The isolate was routinely grown on MA plates at 28 °C and maintained at −80 °C with 25 % (v/v) glycerol.

Biomass for chemotaxonomic and molecular studies was obtained following growth in shake flasks with MB at 28 °C for 2 days. *Bacillus azotoformans* DSM 1046^T, *Bacillus methanolicus* DSM 16454^T and *Bacillus subtilis* subsp. *subtilis* DSM 10^T (included as the type species of the genus *Bacillus*) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DMSZ; Germany) and selected for physiological, biochemical and chemotaxonomic characteristics comparison.

Morphological, physiological and biochemical characterisation

Cell morphology and motility were observed with an optical microscope (BX40; Olympus) and by transmission electron microscopy (JEM-1230; JEOL) (Huo et al. 2010). Gram staining was performed by following the method outlined by Dong and Cai (2001). To determine the growth conditions of strain KC1^T, 0–15 % (w/v) NaCl concentrations with intervals of 1 % were used. The ranges of pH and temperature for growth were determined according to Zhang et al. (2013).

Catalase and oxidase activities, nitrate and nitrite reduction were tested according to Dong and Cai (2001). Indole, methyl red and Voges-Proskauer tests, H_2S production as well as hydrolysis of aesculin, casein, gelatin and starch were performed as described by Zhu (2011). To analyze the use of accessory electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (5 mM), sodium nitrite (5 mM) and sodium nitrate (20 mM) were respectively added to the sterile MB. Oxygen was removed as described by Grishchenkov et al. (2000). The same medium lacking L-cysteine and resazurin was used as aerobic control. Other enzyme activities, physiological and biochemical characteristics were further determined by using the API ZYM and API 20NE kits, using distilled water supplemented with 4 % (w/v) NaCl to suspend the cells. Acid production tests were determined using API 50CH kits, using modified MB from which yeast extract and peptone were omitted and 0.05 g/L yeast extract was added. Utilisation of single carbon sources for growth was carried out using Biolog GP2 96-well Microplates (Biolog) for the oxidation of 95 organic carbon sources in modified MB. All API tests were performed according to the manufacturer's instructions (bioMérieux). Antibiotic sensitivity tests were determined on MA plates with antibiotic discs containing the following amounts (μg per disc, unless indicated): amikacin (30), amoxicillin (10), bacitracin (0.04 IU), cefoxitin (30), cefotaxime (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), neomycin (30), nitrofurantoin (300), norfloxacin (10), novobiocin (30), nystatin (100), polymyxin (300 IU), rifampicin (5), sulfamethoxazole (300), tobramycin (10) and vancomycin (30).

Chemotaxonomic characterisation

For fatty acid methyl esters (FAMEs) analysis, late exponential phase cells of strains KC1^T, *B. azotoformans* DSM 1046^T, *B. methanolicus* DSM 16454^T and *B. subtilis* subsp. *subtilis* DSM 10^T were harvested from MB. FAMEs were obtained from freeze-dried cells as described by Kuykendall et al. (1988) and their identification and quantification were performed using the Sherlock Microbial Identification System (MIDI) with the standard MIS Library Generation Software version 4.5 (Microbial ID). Polar lipids of strain KC1^T and the three reference strains were extracted and examined by TLC on silica gel 60 F_{254} aluminium-backed thin-layer plates (10 × 10 cm, Merk 5554) and further analysed using previously described procedures (Minnikin et al. 1984). The solvent system chloroform/methanol/water (65:24:4, by vol.) was used in the first dimension and chloroform/glacial acetic acid/methanol/water (80:15:12:4, by vol.) was used in the second dimension. The TLC plates were sprayed with sulfuric acid/ethanol (1:1, v/v) and heated at 120 °C for 10 min to reveal total lipids. Isoprenoid quinones and the isomer type of the diamino acid in the cell wall peptidoglycan were determined as described by Komagata and Suzuki (1987).

Molecular studies

The 16S rRNA gene was amplified and analysed as described previously (Xu et al. 2007). PCR products were cloned into the vector pMD19-T (TaKaRa) and then sequenced. The sequence was submitted to GenBank to search for similar sequences by using EzTaxon-e server (Kim et al. 2012) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments with sequences of the most closely related bacteria and calculations of levels of sequence similarity were carried out using CLUSTAL W1.8 (Thompson et al. 1994). Phylogenetic analyses were performed using the following tree-making algorithms: the neighbour-joining (Saitou and Nei 1987), the maximum-parsimony (Fitch 1971) and the maximum-likelihood (Felsenstein 1981) methods using the MEGA 5 program package (Tamura et al. 2011). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura 1980) for the neighbour-joining method. The DNA G+C content was determined by reversed-phase HPLC as described by Mesbah and Whitman (1989).

Results and discussion

Morphological, physiological and biochemical characteristics

Colonies of strain KC1^T on MA plate after 2 days incubation were observed to be 0.5–1.5 mm in diameter, cream, circular and elevated. The cell morphology of strain KC1^T is shown in Fig. S1. Cells were observed to be Gram-positive, rod-shaped (0.5–0.9 × 1.7–4.6 μm), endospore-forming and motile with flagella. Catalase and oxidase activities, methyl red test, H_2S production and hydrolysis of casein were found to be positive. Indole and Voges-Proskauer tests, hydrolysis of starch and gelatin, nitrate reduction, as well as the production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease were found to be negative. The physiological and biochemical characteristics of strain KC1^T that differentiate it from the reference strains *B. azotoformans* DSM 1046^T, *B. methanolicus* DSM 16454^T and *B. subtilis* subsp. *subtilis* DSM 10^T are shown in Table 1. The detailed physiological and biochemical characteristics of strain KC1^T are given in the species description. Strain KC1^T was found to be resistant to bacitracin, nystatin and polymyxin but sensitive to amikacin, amoxicillin, cefoxitin, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, neomycin, nitrofurantoin, norfloxacin, novobiocin, rifampicin, sulfamethoxazole, tobramycin and vancomycin.

Chemotaxonomy results

The major fatty acids of strain KC1^T were identified as anteiso-$C_{15:0}$ (56.6 %), anteiso-$C_{17:0}$ (11.1 %), iso-$C_{15:0}$ (4.6 %), iso-$C_{17:0}$ (4.6 %), $C_{18:0}$ (4.5 %) and $C_{16:0}$ (4.2 %); the complete fatty acid profiles of strain KC1^T and the three reference strains are summarised in Table 2. The major polar lipids were identified as phosphatidylglycerol and a glycolipid, whereas *B. methanolicus* DSM 16454^T and *B. subtilis* subsp. *subtilis* DSM 10^T contained diphosphatidylglycerol in addition to phosphatidylglycerol and glycolipid. *B. azotoformans* DSM 1046^T contained diphosphatidylglycerol, phosphatidylethanolamine and minor amounts of glycolipid which is different from strain KC1^T (Fig. S2). MK-7 was found to be the predominant menaquinone. The cell wall peptidoglycan was found to contain *meso*-diaminopimelic acid as the diamino acid.

Table 1 Differential characteristics among strains KC1^T, *B. azotoformans* DSM 1046^T, *B. methanolicus* DSM 16454^T and *B. subtilis* subsp. *subtilis* DSM 10^T

Characteristics	1	2	3	4
Colony colour	Cream	Translucent[a]	Pale orange[c]	Cream[c]
Colony size (mm)	0.5–1.5	5.0[a]	1.0–2.0[c]	1.0–2.5[c]
pH range	6.5–9.0	5.0–7.0[b]	6.0–6.5[c]	5.0–8.0[c]
Temperature range (°C)	25–45	20–35[b]	35–60[c]	15–55[c]
NaCl range (%)	0–10.0	0–8.0[b]	0–4.5[c]	0–7.0[c]
Motility	+	+[a]	−[c]	+[c]
Arginine dihydrolase	−	−	+	+
Hydrolysis of casein	+	−	−	−
Hydrolysis of gelatin	−	−	+	−
Nitrate reduction to nitrites	−	−	+	+
Acid production from				
N-Acetylglucosamine	+	−	−	+
Adipic acid	−	+	−	−
Amygdalin	−	−	−	+
D-Arabinose	−	−	−	+
L-Arabinose	+	−	−	+
Arbutin	−	−	−	+
D-Cellobiose	−	−	−	+
D-Fucose	−	+	+	+
Gentiobiose	−	−	−	+
D-Glucose	−	−	+	+
Glycerol	−	−	−	+
Glycogen	−	+	+	+
Lactic acid	−	−	−	+
Maltose	+	−	+	+
D-Mannose	+	−	−	+
D-Mannitol	+	−		+
D-Melibiose	−	−	−	+
D-Raffinose	−	−	−	w
D-Ribose	−	−	+	+
Salicin	−	−	−	+
D-Sorbitol	−	+	−	+
Sucrose	+	+	−	+
D-Tagatose	−	−	+	−
D-Trehalose	+	+	−	+
D-Turanose	−	+	+	w
D-Xylose	+	+	−	+
Enzyme activity				
Alkaline phosphatase	+	+	−	+
Acid phosphatase	+	+	−	+
α-chymotrypsin	−	+	−	−
Cystine arylamidase	+	+	−	−
Esterase	+	+	w	+
Esterase lipase	+	+	−	+

Table 1 continued

Characteristics Colony colour	1 Cream	2 Translucent[a]	3 Pale orange[c]	4 Cream[c]
α-galactosidase	−	−	−	+
β-galactosidase	−	−	−	+
α-glucosidase	+	+	−	+
β-glucosidase	−	−	−	+
Leucine arylamidase	+	+	w	w
Lipase	w	+	−	−
Trypsin	−	−	−	w
Valine arylamidase	−	+	−	−
Ciprofloxacin (5 μg)	Sensitive	Sensitive	Resistant	Sensitive
Norfloxacin (10 μg)	Sensitive	Sensitive	Resistant	Sensitive
DNA G+C mol content (%)*	39.0	39.8	48.2	46.6
Major polar lipids	PG, GL	PG, DPG, PE, PL	PG, DPG, GL	PG, DPG, GL
Major fatty acids (>10 %)	anteiso-$C_{15:0}$, anteiso-$C_{17:0}$	iso-$C_{14:0}$, iso-$C_{15:0}$ anteiso-$C_{15:0}$	anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$	anteiso-$C_{15:0}$, iso-$C_{15:0}$

Taxa 1 strain KC1^T, *2 B. azotoformans* DSM 1046^T, *3 B. methanolicus* DSM 16454^T, *4 B. subtilis* subsp. *subtilis* DSM 10^T. Data of these four strains were obtained in this study except were otherwise indicated. − negative; + positive; *w* weakly positive

[a, b, c] For strains *B. azotoformans* DSM 1046^T, *B. methanolicus* DSM 16454^T, *B. subtilis* subsp. *subtilis* DSM 10^T were taken from Pichinoty et al. (1983), Lee et al. (2014) and Pham and Kim (2014), respectively

Data marked with *asterisk* indicated the DNA G+C contents of four strains were determined by HPLC in the same condition

Molecular characterisation

The almost complete 16S rRNA gene sequence (1509 bp) of strain KC1^T was obtained (GenBank accession number KF751884). Based on 16S rRNA gene sequence analysis, strain KC1^T was found to show high sequence similarities with *B. azotoformans* DSM 1046^T (95.4 %) and *B. methanolicus* DSM 16454^T (95.4 %), followed by *B. horikoshii* DSM 8719^T (95.3 %), *B. andreesenii* DSM 23947^T (95.2 %), *B. bataviensis* DSM 15601^T (95.1 %) and *B. drentensis* DSM 15600^T (95.0 %). Levels of 16S rRNA gene sequence similarity between strain KC1^T and other species of the genus *Bacillus* were found to be below 95 %. Phylogenetic trees revealed that strain KC1^T clusters within the genus *Bacillus* and to be closely related to *B. azotoformans* DSM 1046^T and *B. methanolicus* DSM 16454^T (Fig. 1, Figs. S3 and S4). The genomic G+C content of strain KC1^T was determined to be 39.0 mol%.

Taxonomic conclusion

The following observations support the conclusion that strain KC1^T belongs to the genus *Bacillus*: strain KC1^T is Gram-positive, motile, rod-shaped and endospore-forming; the major fatty acid is anteiso-$C_{15:0}$; the major polar lipid is phosphatidylglycerol; the predominant menaquinone is MK-7 and the cell wall peptidoglycan diamino acid is *meso*-diaminopimelic acid; strain KC1^T was phylogenetically clustered within the genus *Bacillus* and closest to strains *B. azotoformans* DSM 1046^T and *B. methanolicus* DSM 16454^T; the G+C content of the genomic DNA was determined to be 39.0 mol%. However, strain KC1^T shows significant different physiological and biochemical characteristics from the type strains of the most closely related species *B. azotoformans* DSM 1046^T and *B. methanolicus* DSM 16454^T, and compared with *B. subtilis* subsp. *subtilis* DSM 10^T, with regard to morphological features, growth conditions (temperature, pH and NaCl ranges), hydrolysis of casein and gelatin, acid production from carbohydrates, enzyme activity and antibiotic sensitivities (Table 1). Moderate differences in fatty acid composition were also observed between strain KC1^T and the three reference species. The amount of anteiso-$C_{15:0}$ in strain KC1^T was notably higher than in the three reference strains and the amount of iso-$C_{15:0}$ was notably lower in the three reference strains. The

Table 2 Cellular fatty acid compositions (%) of strains KC1^T, *B. azotoformans* DSM 1046^T, *B. methanolicus* DSM 16454^T and *B. subtilis* subsp. *subtilis* DSM 10^T

Fatty acid	1	2	3	4
Saturated fatty acids				
$C_{14:0}$	1.2	2.2	tr	tr
$C_{16:0}$	4.2	3.1	2.5	2.4
$C_{18:0}$	4.5	–	tr	tr
$C_{20:0}$	3.0	–	–	–
Branched fatty acids				
iso-$C_{14:0}$	2.7	33.4	1.4	1.3
iso-$C_{15:0}$	4.6	29.2	27.6	27.9
anteiso-$C_{15:0}$	56.6	21.7	35.8	40.3
iso-$C_{16:0}$	1.4	5.9	2.6	2.6
iso-$C_{17:0}$	4.6	tr	10.3	8.5
anteiso-$C_{17:0}$	11.1	tr	9.1	9.1
iso-$C_{18:0}$	1.0	–	–	–
iso-$C_{19:0}$	1.4	–	–	–
anteiso-$C_{19:0}$	2.7	–	–	–
Hydroxy fatty acids				
$C_{15:0}$ 2-OH	–	–	2.4	1.5
iso-$C_{15:0}$ 3-OH	–	–	3.1	1.7

Taxa 1 strain KC1^T, *2 B. azotoformans* DSM 1046^T, *3 B. methanolicus* DSM 16454^T, *4 B. subtilis* subsp. *subtilis* DSM 10^T. Values are percentages of the total fatty acid content

tr trace component (<1 %); – not detected. (All data were obtained in this study under the same experimental conditions)

detailed fatty acid profiles of the four strains are displayed in Table 2. Differences in the polar lipid profiles were also found among the four strains as shown in Fig. S2. Moreover, the 16S rRNA gene sequence analysis indicated that strain KC1^T shared lower than 96 % sequence similarities to other validly named species in genus *Bacillus*.

Based on the phenotypic, chemotaxonomic and genotypic characteristics described above, strain KC1^T can be concluded to represent a novel species within genus *Bacillus*, for which the name *Bacillus salitolerans* sp. nov. is proposed.

Description of *Bacillus salitolerans* sp. nov.

Bacillus salitolerans (sa.li.to'le.rans. L. n. *sal*, *salis* salt; L. part. adj. *tolerans* tolerating; N.L. part. adj. *salitolerans* salt tolerating).

Cells are Gram-positive, strictly aerobic, motile by means of flagella, rod-shaped (0.5–0.9 × 1.7–4.6 μm) and endospore-forming. Colonies are 0.5–1.5 mm in diameter, cream, circular and elevated on MA plates after 2 days incubation. Growth occurs at 25–45 °C (optimum 37 °C), pH 6.5–9.0 (optimum 8.0) and NaCl 0–10 % (v/v) (optimum 4 %). Growth does not occur under anaerobic conditions with sodium thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite and sodium nitrate as electron acceptors. Catalase and oxidase activities, methyl red test, H_2S production and hydrolysis of casein are positive. Indole and Voges-Proskauer tests, nitrate reduction, hydrolysis of starch and gelatin, as well as the production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease are negative. Positive in the Biolog GP2 MicroPlate for the oxidation of acetic acid, *N*-acetylglucosamine, adenosine-5′-monophosphate, L-alaninamide, L-alanine, L-alanyl-glycine, amygdalin, D-araviyol, L-arabinose, arbutin, L-asparagine, 2, 3-butanediol, D-cellobiose, β-cyclodextrin, L-fructose, D-fructose, D-fructose-6-phosphate, D-galactose, gentiobiose, D-glucose, α-D-glucose-1-phosphate, D-glucose-6-phosphate, L-glutamic acid, D-L-α-glycerol phosphate, glycerol, glycogen, α-hydroxybutyric, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic, *m*-inositol, inulin, α-ketoglutaric acid, lactamide, D-lactic acid methyl ester, α-D-lactose, lactulose, D-malic acid, L-malic acid, maltose, mannan, D-mannitol, D-mannose, D-melibiose, β-methyl-D-galactoside, 3-methyl-glucose, palatinose, propionic acid, D-psicose, putrescine, pyruvic acid, L-pyroglutamic acid, D-raffinose, L-rhamnose, sedoheptulosan, D-sorbitol, succinamic acid, succinic acid, succinic acid mono-methyl ester, thymidine, thymidine-5′-monophosphate, trehalose, Tween 80 and uridine. Other substrates in the Biolog GP2 MicroPlate are negative. According to the API 50 CH system, positive for acid production from *N*-acetyl-β-D-glucosamine, aesculin, L-arabinose, D-fructose, 2-ketogluconate, 5-ketogluconate, maltose, D-mannitol, D-mannose, α-methyl-D-glucopyranoside, sucrose, trehalose and D-xylose; other substrates of the API 50 CH system are not converted to acid. With the API ZYM and API 20 NE systems, acid phosphohydrolase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), α-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase are positive. Lipase (C14) is weakly positive. *N*-acetyl-β-glucosaminidase, α-chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, β-

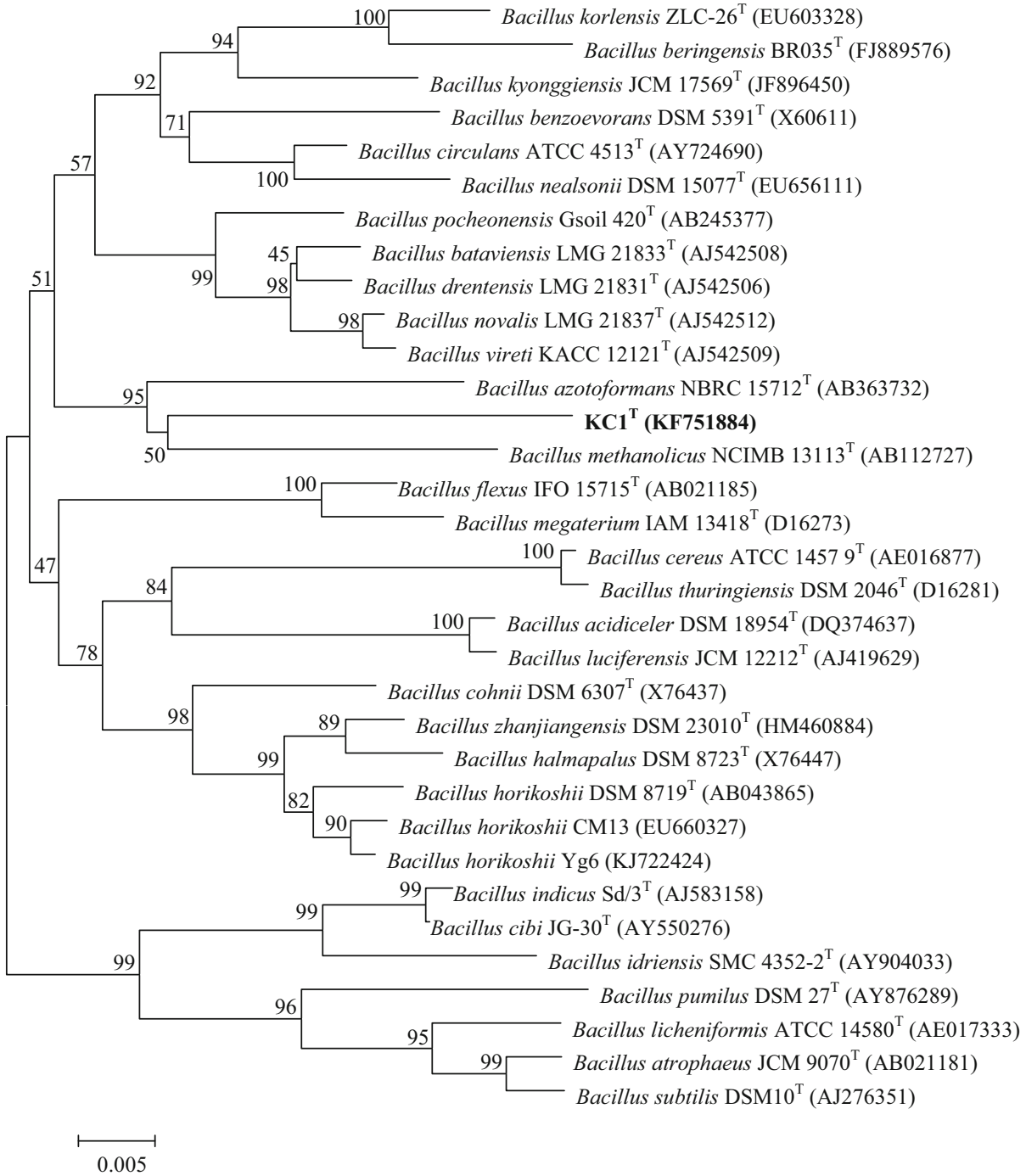

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequences of strains KC1^T and its taxonomic neighbours constructed with the neighbour-joining method. Bootstrap values (>50 %) based on 1000 replications are listed as percentages at branching points. *Bar* 0.005 substitutions per nucleotide position

glucuronidase, α-mannosidase, trypsin and valine arylamidase are negative. The major fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. Menaquinone-7 (MK-7) is the predominant isoprenoid quinone. The cell wall peptidoglycan contains *meso*-diaminopimelic acid as the diamino acid. Major polar lipids are

phosphatidylglycerol and a glycolipid. The DNA G+C content of the type strain is 39.0 mol% (HPLC).

The type strain KC1^T (=JCM 19760^T = CGMCC 1.12810^T) was isolated from a salt mine taken from Kuche county, Xinjiang province, China. The GenBank accession number of the 16S rRNA gene sequence of the type strain is KF751884.

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