Lysobacter Chinensis sp. nov., a Cellulose-degrading Strain Isolated From Cow Dung Compost

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Abstract

A novel bacterial strain, TLK-CK17^T, was isolated from cow dung compost sample. The strain was Gram-stain-negative, non-spore-forming, short rod-shaped, aerobic, and displayed growth at 15–40°C with an optimum at 35°C, with 0–5.0% (w/v) NaCl (optimum 0.5) and at pH 6.5–8.5 (optimum 7.0–7.5). Cells contained summed iso-$C_{16:0}$, iso-$C_{15:0}$, and feature 9 (comprising C17:1 *ω*9*c* and / or 10-methyl $C_{16:0}$), as its major cellular fatty acids (>10.0%) and ubiquinone-8 (Q-8) as the exclusively respiratory quinone. The polar lipid profile of strain TLK-CK17^T consisted of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content was 68.2 mol%. On the basis 16S rRNA gene sequence comparison, strain TLK-CK17^T showed the highest sequence similarity (98.9%) with *L. penaei* GDMCC 1.1817^T, followed by *L. maris* KCTC 42381^T (98.3%). Furthermore, the average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) between strain TLK-CK17^T and closely related strains *L. penaei* GDMCC 1.1817^T, and *L. maris* KCTC 42381^T were 79.9–85.6% and 23.9–29.6%, respectively. Based on presented results, we propose a novel species for which the name *Lysobacter chinensis* sp. nov. is suggested, with the type strain TLK-CK17^T (=CCTCC AB2021257^T= KCTC 92122^T).

Introduction

The genus *Lysobacter* was proposed by Christensen and Cook (1978) and from the family *Lysobacteraceae*, phylum '*Proteobacteri*'. As of December 2021, the genus includes 85 validly published species with correct name; the type species is *Lysinimonas soli* (Jang et al. 2013). Members of the genus *Lysobacter* commonly known to be a Gram-negative, rod-shaped with pink to yellow colonies, and contain high DNA G+C content (61.7–70.1 mol%) (Christensen 2005; Li et al. 2018). Strains of *Lysobacter*, thought to play vital roles in the environment for their high enzyme production capacity, are ubiquitous in various ecosystems. At present, they have been isolated from various habitats, including diferent types of soil such as cave soil (Chen et al. 2016), forest soil (Margesin et al. 2018), cultivated soil (Siddiqi and Im 2016), manganese factory soil (Li et al. 2018), arid area soil (Lee et al. 2017a), and abandoned gold mine (Liu et al. 2011). Here, we report a polyphasic taxonomical description of a novel, cellulose-degrading bacterium strain, designated TLK-CK17^T. The phenotypic, chemotaxonomic and genotypic properties indicate that strain TLK-CK17^T represents a novel species within the genus *Lysobacter*, for which the name *Lysobacter chinensis* sp. nov. is proposed.

Materials And Methods

Isolation and cultivation

In our investigation of the diversity of cultured bacteria in cow dung compost, Xinjiang Uygur Autonomous Region, China (43°81'N, 87°57'E), strain TLK-CK17^T was isolated. The compost sample was suspended in sterile water and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, then 100 µl from each dilution was spread onto 1/3 nutrient agar (NA) plates. The plates were incubated at 30°C and checked for growth

after 2-3 days. After incubation, a single colony was purified by sub-culturing under the same conditions. For further investigation, the isolate was sub-cultured on NA plates at 30°C and preserved at −80°C as glycerol suspension (20%, w/v). Meanwhile, closely related strains *L. penaei* GDMCC 1.1817^T, and *L. maris* KCTC 42381^T were obtained from the Guangdong Microbial Culture Collection Center (GDMCC) and the Korean Collection for Type Culture, respectively.

DNA extraction and genome sequencing

Genomic DNA of strain TLK-CK17^T was extracted and purified using a bacterial genomic DNA kit (Takara), following the manufacturer's recommendations. The taxonomic position of strain TLK-CK17^T was first determined by 16S rRNA gene sequence using the primers XJ11 (5'-AGAGTTTGATCCTGGCTCAG-3') and XJ22 (5'-GGTTACCTTGTTACGACTT-3'). Whole-genome sequencing was performed on the Illumina HiSeq PE150 platform. A-tailed, ligated to paired-end adaptors and PCR amplified with a 350 bp insert was used for the library reconstruction. The raw reads were assembled using SOAPdenovo version 2.04 software (Li et al. 2008; Li et al. 2010). The genes of strain TLK-CK17^T were identified by NCBI Prokaryotic Genome Annotation Pipeline server online and the Pfam database (Angiuoli et al. 2008; http://pfam.xfam.org/), and the genes involved in metabolic pathways were analyzed in detail using the information present in RAST (Rapid Annotation using Subsystem Technology; https://rast.nmpdr.org). The G+C content of the chromosomal DNA was calculated using genome sequencing. The digital DNA–DNA hybridization (dDDH) values were calculated using the Genome-to-Genome Distance Calculator (GGDC 2.0) (Meier-Kolthoff et al. 2013). The average nucleotide identity (ANI) values were calculated using the algorithm of Goris *et al*. (Goris et al. 2007) by using the EzGenome web service.

Processing of sequence data and phylogenetic analysis

The complete 16S rRNA sequence of strain TLK-CK17^T was uploaded to the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) and the NCBI GenBank to indentify the strain based on the sequences available. Multiple alignments with corresponding sequences of the closely related strains were aligned using CLUSTAL_X (Thompson et al. 1997). Phylogenetic analysis was conducted by neighbour-joining (NJ) (Saitou et al. 1987), maximum-parsimony (MP) (Fitch et al. 1971) and maximum-likelihood (ML) (Felsenstein et al. 1981) methods in MEGA 7.0 program (Kumar et al. 2016) using the Kimura two-parameter model (Kimura et al. 1980), and the gaps were treated using a partial deletion method. Bootstrap analysis with 1000 replications was conducted, aimed at estimating the topology of the phylogenetic tree (Felsenstein et al. 1985).

Phenotypic and biochemical characteristics

Gram-staining and morphological features were tested with cells grown on NA plates at 35°C for 24 h. Gram-staining was performed using a Gram stain kit (bioMérieux) according to the manufacturer's instructions. Cell morphology and size were examined with light microscopy (E600; Nikon) and transmission electron microscopy (JEM-1200; JEOL). Motility was determined using the hanging-drop method and gliding motility was determined as described by Bowman (Bowman et al. 2000). Reduction

of nitrate was performed as described by Cowan and Steel (Cowan et al. 1974). Catalase, oxidase and lipase (Tweens 20 and 80) activities and hydrolysis of starch and CM-cellulose were tested according to the methods of Dong and Cai (Dong and Cai et al. 2001). Anaerobic growth was tested after incubation for 2 weeks at 35°C on NA with or without 0.1% (w/v) KNO_3, in an anaerobic chamber filled with a gas mixture (10% H_2, 10% CO_2 and 80% N_2). Growth at different temperatures (0, 4, 10, 15, 20, 28, 30, 33, 37, 40, 45 and 50°C) and at different concentrations of NaCl (0, 0.5, 1, 2, 3, 4, 5 and 6%, w/v) were investigated on NA for up to 10 days. Growth at pH 5.5–9.5 (at intervals of 0.5 pH unit) was determined by measuring the optical density (wavelength 600 nm) of cultures in NB with the pH adjusted prior to sterilization by adding the appropriate buffers, including MES (for pH 5.5 and 6.0), PIPES (for pH 6.5 and 7.0), HEPES (for pH 7.5 and 8.0), Tricine (for pH 8.5) and CAPSO (for pH 9.0 and 9.5). Antibiotic sensitivity was assessed as described by the Clinical and Laboratory Standards Institute (CLSI et al. 2012), and inoculated plates were incubated at 35°C for up to 48 h. Other physiological and biochemical characteristics of strain TLK-CK17^T and closely related strains were determined using the API 20E, API ZYM and API 50CHB identification systems (bioMérieux) and the Biolog GEN III identification system, according to the manufacturers' instructions.

Chemotaxonomic analysis

Strain TLK-CK17^T and two closely related strains were done in parallel with in this fatty acid methyl esters (FAME) analysis when the bacterial communities reached the late exponential stage of growth according to the four quadrants streak method. The cellular fatty acid methyl esters were prepared and identified according to the Microbial Identification System (Sherlock version 4.5; database: TSBA40; MIDI; Sasser et al. 1990). The isoprenoid quinone strain TLK-CK17^T and two closely related strains were extracted from freeze-dried cell material using the two-stage method described by Tindall *et al.* (Tindall et al. 2007) and subsequently analysed by HPLC (Kroppenstedt et al. 1982). Polar lipids were extracted using a chloroform/methanol system and analysed by using two-dimensional thin-layer chromatography, as described previously (Fang et al. 2017; Minnikin et al. 1984).

Results And Discussion

Phylogenic analysis

According to the comparisons with the complete 16S rRNA gene sequence (1545 bp) in the EzTaxon database, highest level of sequence similarity occurred with *L. penaei* GDMCC 1.1817^T (98.9%) and *L. maris* KCTC 42381^T (98.3%). The phylogenetic position of the novel isolate, determined using various tree-making algorithms, confrmed that strain TLK-CK17^T was a member of the genus *Lysobacter*, forming a coherent cluster with the two abovementioned members of this genus in the NJ, ML and MP trees with low bootstrap values, respectively (Fig. 1). On the basis of 16S rRNA gene sequence phylogenetic analysis, two strains *L. penaei* GDMCC 1.1817^T and *L. maris* KCTC 42381^T were chosen as reference strains in this study.

Phenotypic and biochemical characterisation

NA medium was used for general laboratory cultivation, but the novel strain also grows well on TSA and R_2A media. After 24 h growth on NA at 30 C, colonies were observed to be 1.0–1.5 mm in diameter, circular, smooth and apricot. Strain TLK-CK17^T was found to be Gram-reaction-negative and catalase negative aerobic bacterium. Meanwhile, it showed a positive reaction for catalase (weakly) and nitrate reduction. Cells are non-motile, grow in 0–5.0% (w/v) NaCl, at a pH range from 6.5 to 8.5 and at temperatures between 15 and 40°C. Optimal growth was observed at 35°C, 0.5% (w/v) NaCl and pH 7.0-7.5. Cells of strain TLK-CK17^T are short rods, catalase-negative but oxidase-positive, the mean cell size is 0.3–0.5 μm in width and 1.5–2.0 μm in length (Supplementary Fig. 2). Nitrate can be reduced to nitrite. The strain was positive for the hydrolysis of Tween 20, 80, casein, CM-cellulose, but negative for alginate, starch. Antibiotic susceptibility test indicated that the strain was sensitive to chloramphenicol (30 μg), ceftriaxone (30), Ofloxacin (5 μg) and Ciprofloxacin (5 μg). However, it was resistant to penicillin (10 μg), tetracycline (30 μg), vancomycin (30 μg), ampicillin (10 μg), streptomycin (10 μg), Clindamycin (2 μg), Amoxicillin (25 μg) and Cephalexixn (30 μg)

Chemotaxonomic characteristics

The predominant cellular fatty acids of strain TLK-CK17^T was iso-$C_{16:0}$ (24.3%), iso-$C_{15:0}$ (23.8%), and feature 9 (comprising C17:1 ω9*c* and / or 10-methyl $C_{16:0}$, 15.4%). The fatty acid profile was similar to that of closely related strains *L. penaei* GDMCC 1.1817^T, and *L. maris* KCTC 42381^T, in accordance with the description of *Lysobacter* genus. However, the ratios of the different components are different. The complete fatty acid composition was shown in Table 2. Ubiquinone-8 (Q-8) as the exclusively respiratory quinone. Strain TLK-CK17^T exhibited a complex polar lipid profile consisting of Phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) as dominant elements, and one uncharacterised lipid (L) (Supplementary Fig. 3).

Whole genome sequence analysis

The genome contributes to an important understanding for the genetic evolution of bacteria, disease prevention and treatment, and the development of antibiotics; thus, the genome of strain TLK-CK17^T was analysed to decipher the genetic code involved in the environmental suitability. The genome size and G + C content of TLK-CK17^T are 4,300,099 bp and 68.2 mol%, respectively. A length of 3,695,277-bp genes was found based on gene prediction, and the ratio of gene length/genome was 85.9%. Furthermore, 3792 CDSs were contained in the genome, and all were assigned functions. CDSs were further annotated in NR, Swiss-Prot, COGs, KEGGs, GO and Pfam, and their numbers were 3630, 1485, 2762, 3421, 2419 and 2419, respectively. The ANI values of strains TLK-CK17^T with *L. penaei* GDMCC 1.1817^T and *L. maris* KCTC 42381^T were 79.9% and 85.6%, respectively, while the GGDC values were 29.6 and 23.8, respectively. For species delineation, ANI values of 95–96% and dDDH values of 70%, respectively, are normally accepted (Wayne et al. 1987; Thompson et al. 2013). These results indicated that strain TLK-CK17^T represents a

novel species of the genus *Lysobacter*. The related genome datas strain TLK-CK17^T and two closely related type strains are listed in Tables S1 and S2.

The RAST analysis revealed the presence of 302 subsystems, the subsystem coverage was 25% (total 977, non-hypothetical 936, hypothetical 41), and 67 glycoside hydrolase (GHs), 42 glycosyl transferases (GTs), 44 carbohydrate-binding modules (CBMs), 6 Carbohydrate Esterases (CEs), and 4 auxiliary activities (AAs) were identified. Because cow dung is rich in lignocellulose, the coexistence of these genes suggest that they play important roles in the breakdown and modification of carbohydrates in cow dung composting. The assembled genome of strain TLK-CK17^T was compared to the genomes of closely related strains *L. penaei* GDMCC 1.1817^T and *L. maris* KCTC 42381^T. Result showed that GH, GT and CBM family numbers were more in strain TLK-CK17^T, while the *L. penaei* GDMCC 1.1817^T contained more CE and AA family members (Table S2). We considered that it might be closely related to their isolated environment, *L. penaei* GDMCC 1.1817^T and *L. maris* KCTC 42381^T were isolated from the pacific white shrimp and seawater, respectively.

Lysobacter spp. has been identified as heterotrophic with a wide range of extracellular enzymes and other metabolites against other microorganisms, including fungi and nematodes, so it played an important role in the suppression of pathogenic bacteria (de Bruijn *et al*. 2015; Xie et al. 2012; Pidot et al. 2014). Our results showed that the strain TLK-CK17^T possessed chitinase, protease and glucanase activity, confirming and extending previous research (Zhang et al. 2001; Palumbo et al. 2005). Chitinase, glucanase and protease activities may contribute to antimicrobial activity, since chitin, α- and β-glucans and glycoproteins are the major components of the cell walls of fungi (Figueiredo et al. 2014). Moreover, we analysed that *Lysobacter* strains showed a high genetic diversity, which could confer an advantage under adverse environmental conditions (Foster et al. 2005). To better understand the potential effect of strain TLK-CK17^T to the overall activities of the microbial communities in cow dung compost, our future work will include testing it with other bacterial genera abundant in compost. Interactions of strain TLK-CK17^T with other bacteria whether or not stimulate the production of antimicrobial compounds, so as to quickly remove pathogenic microorganisms in livestock manure.

Based on the phylogenetic analyses, strain TLK-CK17^T was found to be affiliated with members of the genus *Lysobacter* in the family *Xanthomonadaceae*. Strain TLK-CK17^T represented a novel species of the genus *Lysobacter*, as supported by the related genome datas. In addition, phenotypic and biochemical collectively support the fact that strain TLK-CK17^T was distinguishable, whilst chemotaxonomic analyses are consistent with their affiliation with the genus *Lysobacter*. The phenotypic characterisation presented in Table 1 differentiates strain TLK-CK17^T as a separate species. The exclusively respiratory quinone was Q-8, as reported for the major respiratory quinone of all members of the genus *Lysobacter*. The major fatty acids of type strain TLK-CK17^T were iso-$C_{16:0}$, iso-$C_{15:0}$, and feature 9 (comprising C17:1 $\omega 9c$ and / or 10-methyl $C_{16:0}$). Based on the results presented in this study, it is proposed that strain TLK-CK17^T represents novel member in the genus *Lysobacter*, for which the names *Lysobacter chinensis* sp. nov., is

proposed. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and whole-genome shotgun project of strain TLK-CK17^T is OK143236.

Description of *Lysobacter chinensis* sp. nov.

Lysobacter chinensis (chi.nen'sis. N.L. masc. adj. *chinensis* pertaining to China, where the type strain was isolated)

Cells are Gram-stain-negative, aerobic, non-motile, and short rod-shaped with a size of 0.3–0.5×1.5–2.0 μm. Good growth is observed on R2A agar, TSA and NA, but not on MA. Colonies on NA are beige to apricot, smooth, opaque, circular (approximately 1.0-1.5 mm in diameter) with entire edges and convex. Growth occurs on NA at temperatures of 15–40°C (optimum 35°C). The pH range for growth is from pH 6.5 to 8.5 (optimum pH 7.0–7.5). Growth occurs at 0–5.0% NaCl concentrations (optimum 0.5). Catalase-positive and oxidase-negative. Nitrate can be reduced to nitrite. CM-cellulose, starch, casein, Tweens 20 and 80 are hydrolysed, but alginate is not. Positive for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase, *α*-glucosidase, and *β*-glucosidase, but negative for esterase lipase (C8), lipase (C14), cystine arylamidase, *α*-chymotrypsin, acid phosphatase, *β*-glucuronidase, *N*-acetyl-glucosaminidase and *α*-mannosidase. Positive for ONPG test, indole production and gelatinase, but negative for H_2S production, Voges-Proskauer reaction, Simmons' citrate utilization. Acid is produced from l-arabinose, d-xylose, d-galactose, d-glucose, d-mannose, amygdalin, ESC, d-cellobiose, d-maltose (weakly), d-lactose, d-melibiose, d-sucrose, d-raffinose, glycogen, d-gentiobiose. Positive for oxidation of d-trehalose, d-cellobiose (weakly), gentiobiose, *N*-acetyl-*β*-d-mannosamine (weakly), d-mannose, d-galactose. The major cellular fatty acids are iso-$C_{16:0}$, iso-$C_{15:0}$, and feature 9 (comprising C17:1 *ω*9*c* and / or 10-methyl $C_{16:0}$). The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The exclusively respiratory quinone is Q-8.

The type strain TLK-CK17^T (=CCTCC AB2021257^T= KCTC 92122^T) was isolated from cow dung compost sample collected from the Xinjiang Uygur Autonomous Region, China (43°81'N, 87°57'E). The genomic DNA G+C content of the type strain was 68.2 mol%.

Abbreviations

KCTC, Korean Collection for Type Culture; CCTCC, China Center for Type Culture Collection; ANI, average nucleotide identity; GGDC, Genome-to-Genome Distance Calculator; MIDI, Microbial Identification System; HPLC, High Performance Liquid Chromatography; PE, phosphatidylethanolamine; PG, phosphatidylglycerol

Declarations

Author contributions

Y.Y.L. wrote the manuscript and analysed the cultivation data, L.Y.Z. performed the genomic and phylogenetic analysis, Y.X.P. and P.B.L. isolated the strain and performed the initial cultivation and strain deposition, Y.X.X and J.P.D. contributed to text preparation and revised the manuscript, L.F performed the electron microscopic analysis and prepared the SEM pictures, X.W.W and Z.F.W. took the samples, supervised A.H. and the study. All authors read and approved the final version of the manuscript.

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Availability of data and material

The genome and 16S rRNA gene sequence are available from GenBank under the accession numbers provided in the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships.

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Tables

Table 1. Physiological and chemotaxonomic properties of strain TLK-CK17^T compared with the closely related species of the genus *Lysobacter*.

Strains: 1, TLK-CK17^T; 2, *L. penaei* GDMCC 1.1817^T; 3, *L. maris* KCTC 42381^T. Data were obtained in the present study unless indicated. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristics	1	2	3
Isolated	Cow dung compost	Pacific white shrimp	Rhizosphere of pepper
Pigmentation	Apricot	light yellow	Apricot
NaCl range for growth (%)	0-5.0	0-6.0	0-7.0
Tween 20	+	+	–
Tween 80	+	–	+
Oxidase	w	+	–
Catalase	–	+	+
API ZYM assay:			
Lipase (C14)	–	–	w
Valine arylamidase	+	w	–
Trypsin	–	–	w
α-Galactosidase	+	–	–
α-Glucosidase	+	–	+
N-Acetyl-β-glucosaminidase	–	–	+
API 20NE assay:			
Reduction of nitrate to nitrite nitrite	+	+	
β-Galactosidase	+	w	w
d-Glucose	+	w	w
l-Arabinose	+	–	–
d-Mannose	w	–	–
N-Acetyl-glucosamine	–	+	+
Maltose	w	+	+
DNA G+C content (mol%)	68.2	68.8	69.0
Genome size (Mb)	4.3	3.2	4.0
Genomic genes	3630	2872	3578

Table 2. Cellular fatty acid compositions of strain TLK-CK17^T and phylogenetically related species of the genus *Lysobacter*.

Strains: 1, TLK-CK17^T; 2, *L. penaei* GDMCC 1.1817^T; 3, *L. maris* KCTC 42381^T. Data were obtained in the present study unless indicated. Values are percentages of the total fatty acids, and only fatty acids comprising >0.5 % are shown. The fatty acids in bold are the major cellular fatty acid (>10.0 %). Results are scored as follows: TR, Trace (<0.5 %); –, not detected.

Fatty acid	**1**	**2**	**3**
$C_{16:0}$	2.3	4.2	2.1
$C_{16:0}$ cyclo	1.6	TR	TR
$C_{17:0}$ cyclo	TR	TR	1.5
iso-$C_{11:0}$	6.5	5.8	6.4
iso-$C_{12:0}$	0.6	TR	TR
iso-$C_{14:0}$	1.1	1.8	1.1
iso-$C_{15:0}$	**23.8**	**20.6**	**20.3**
iso-$C_{16:0}$	**24.3**	**19.3**	**28.4**
iso-$C_{17:0}$	3.1	7.8	5.1
anteiso-$C_{15:0}$	TR	TR	0.8
anteiso-$C_{17:0}$	TR	TR	0.7
iso-$C_{11:0}$ 3-OH	7.8	6.6	7.1
iso-$C_{15:0}$ 3-OH	0.5	TR	TR
iso-$C_{16:1}$ H	1.8	0.5	0.7
***Summed feature**			
1	0.5	TR	TR
3	5.0	8.0	4.3
8	0.7	1.5	1.0
9	**15.4**	**20.2**	**6.0**

Figures

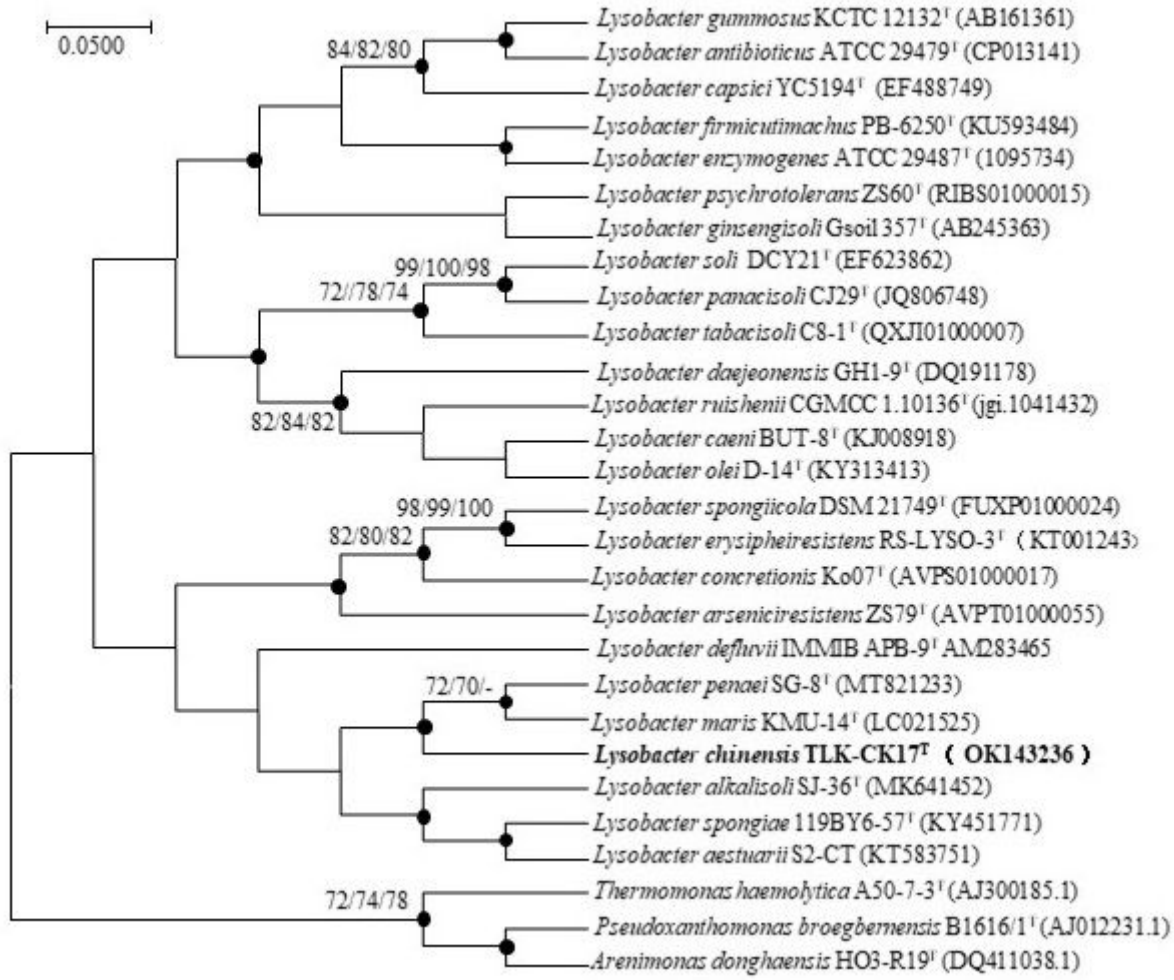

Figure 1

Neighbour-joining phylogenetic tree based on full-length 16S rRNA gene sequence (1545 bp), showing the phylogenetic position of strain TLK-CK17^T among members of the genus *Lysobacter*. Numbers on nodes represent bootstrap values (NJ) based on 1000 replications. Only bootstrap values higher than 70 % are marked on the branches. Filled circles indicate nodes also obtained in both maximum-likelihood and maximum-parsimony trees. Bar, 0.02 substitutions per nucleotide position.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryforTLKCK17.pdf