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Article *in* International Journal of Systematic and Evolutionary Microbiology · February 2019

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Lysobacter psychrotolerans sp. nov., isolated from soil in the Tianshan Mountains, Xinjiang, China

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Abstract

A novel aerobic bacterial strain, designated ZS60^T, with long, rod-shaped, gram-staining-negative, aerobic cells was isolated from the soil in the Tianshan Mountains, Xinjiang, China. Phylogenetic analysis based on its 16S rRNA gene sequence indicated that strain ZS60^T was affiliated with the genus *Lysobacter*, and was most closely related to *Lysobacter daejeonensis* GH1-9^T (96.9 %), *Lysobacter caeni* BUT-8^T (96.8 %) and *Lysobacter ruishenii* CTN-1^T (96.7 %). The average nucleotide identity values between strain ZS60^T, *L. daejeonensis* GH1-9^T and *L. ruishenii* CTN-1^T were 78.14 and 78.39 %, respectively. The DNA–DNA relatedness between strain ZS60^T, *L. daejeonensis* GH1-9^T and *L. caeni* BUT-8^T were 44.8 and 39.1 %, respectively. The genomic DNA G+C content of the strain ZS60^T was 67.7 mol% (draft genome sequence), and Q-8 was the predominant ubiquinone. The major cellular fatty acids of strain ZS60^T were iso-$C_{15:0}$ (23.4 %), iso-$C_{17:0}$ (17.2 %) and iso-$C_{17:1}\omega9c$ (12.6 %). On the basis of genotypic, phenotypic and biochemical data, strain ZS60^T is considered to represent a novel species of the genus *Lysobacter*, for which the name *Lysobacter psychrotolerans* sp. nov. is proposed. The type strain is ZS60^T (=CGMCC 1.15509^T=NBRC 112614^T).

The genus *Lysobacter* was first proposed by Christensen and Cook in 1978 [1]. It belongs to the family *Xanthomonadaceae* of the class *Gammaproteobacteria* based on phyletic classification. Members of the genus *Lysobacter* are Gram-negative, non-fruiting, with high DNA G+C contents typically ranging from 61.7 to 70.1 mol% and contain ubiquinone Q-8 as the major respiratory quinone [2–4]. At the time of writing, the genus comprises more than 40 species with validly published named (www.bacterio.net/), including the recently described species *Lysobacter solanacearum* [5], *Lysobacter humi* [6] and *Lysobacter silvestris* [7]. Some members of the genus were found to have potential for the development of biocontrol agents against micro-organisms such as Gram-negative and Gram-positive bacteria, plant fungal pathogens, and green algae [8–11].

In this paper, strain ZS60^T was isolated from a soil sample collected from Tianshan Mountain, China. Soil samples (5 g) were suspended in sterilized PBS and then gradient-diluted solutions were spread on Reasoner's 2A (R2A) medium (Difco) containing 1.5 % (w/v) agar. After cultivation for 7–20 days at 20 °C, pure colonies on the plates were purified by transferring them onto new plates for an additional incubation for 3 days at 20 °C. This novel strain was then maintained on R2A slants at 4 °C and with 20 % (v/v) glycerol suspensions at −70 °C. The taxonomic statue of strain ZS60^T was investigated by using polyphasic taxonomy.

To analysis the 16S rRNA gene sequence, the genomic DNA of ZS60^T was prepared according to the method of Ausubel *et al.* [12]. Then, the universal bacterial primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) was used for amplification of the 16S rRNA gene as described by Huy *et al.* [13]. After polyacrylamide gel electrophoresis with 1 % (w/v) agarose, the PCR products were sequenced by BGI (China). The almost-complete 16S rRNA gene (1524 bp) was compiled using Seqman software (DNASTAR). The 16S rRNA gene from genome sequencing was under 376–1923 bp in contig 15 (1547 bp). After comparing the 16S rRNA gene sequences from PCR and genome sequencing,

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Keywords: Lysobacter; polyphasic taxonomy; phylogenetic; cellular fatty acids; average nucleotide identity; DNA–DNA relatedness.
Abbreviation: R2A, Reasoner's 2A.
The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain ZS60^T is JQ977478. The whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession RIBS00000000
Two supplementary figures are available with the online version of this article.

the result showed that the two sequences were almost identical. Then, the related taxa of 16S rRNA gene sequences were obtained from GenBank and the EzBioCloud (www.ezbiocloud.net/) [14], among them, *Lysobacter daejeonensis* GH1-9^T (96.9 %), *Lysobacter caeni* BUT-8^T (96.8 %) and *Lysobacter ruishenii* CTN-1^T (96.7 %) had the highest sequence similarity to strain ZS60^T. Multiple alignments were conducted with the CLUSTAL_X program [15]. The phylogenetic tree was reconstructed by using the maximum-likelihood method [16] using the MEGA 7 program [17], 1000 replications were set as a bootstrap of the tree [18].

The G+C content of the genomic DNA was determined by HPLC according to the method of Tamaoka and Komagata [19]. The taxonomic relationships between strain ZS60^T, *L. daejeonensis* GH1-9^T and *L. caeni* BUT-8^T were examined using DNA–DNA hybridization. DNA–DNA hybridization was performed according to the method described by Ezaki *et al.* [20], by using photobiotin-labelled probes and microplate wells. Hybridizations were conducted three times and the three values were used in the calculation of hybridization values. Strain ZS60^T showed low levels of DNA–DNA relatedness with *L. daejeonensis* GH1-9^T (44.8 %) and *L. caeni* BUT-8^T (39.1 %), respectively.

The draft genome sequence of strain ZS60^T were sequenced and assembled by the Marine Culture Collection of China (MCCC) with an Illumina Miseq platform (sequence depth was 200×) and subsequently deposited in the NCBI database under the accession number RIBS00000000. The genome size of strain ZS60^T was 3 910 744 bp with 19 contigs, and the N50 length was 534630 (three contigs). The annotation for ZS60^T was conducted with the NCBI Prokaryotic Genome Annotation Pipeline, a total of 3356 genes were predicted and 3254 coding sequence were identified with 49 tRNA genes and 1, 1, 1 rRNA genes (5S, 16S, 23S), respectively. We also obtained other genome sequences of *L. daejeonensis* GH1-9^T (accession number: AVPU00000000.1) from the NCBI database, and *L. ruishenii* CTN-1^T (accession number: jgi.1041432.1) from the EzBioCloud (www.ezbiocloud.net/) [14]. The average nucleotide identity (ANI) was calculated based on draft genome sequences, by using the ANI calculator (http://www.ezbiocloud.net/tools/ani) programs [21]. The genome sequences of strain ZS60^T showed ANI values of 78.14 and 78.39 % with the sequences of *L. daejeonensis* GH1-9^T and *L. ruishenii* CTN-1^T, respectively. The recommended threshold values for species discrimination (ANI of 95–96 % [22]) indicates that strain ZS60^T should be classified as a distinct species of the genus *Lysobacter*.

Table 1. Differential phenotypic and biomedical characteristics between strain ZS60^T and other related recognized *Lysobacter* species

Strains: 1, ZS60^T (data from this study); 2, *L. daejeonensis* GH1-9^T (data from this study, except for the DNA G+C content which is from Weon *et al.* [31]); 3, *L. caeni* BUT-8^T (data from this study, except for the DNA G+C content which is from Ye *et al.* [32]); 4, *L. ruishenii* CTN-1^T (data from this study, except for the DNA G+C content which is from Wang *et al.* [33]). +, Positive; −, negative; W, weakly positive; ND, not determined. All four strains have no ability to degrade starch.

Characteristic	1	2	3	4
Isolation source	Soil	Plant	Water	Soil
Cell shape	Long rod	Long rod	Long rod	Rod
Size (μm)	0.3–2.3	0.4–2.8	0.5–2.0	0.5–1.6
Growth pH	6.0–10.0	6.0–8.0	5.0–10.0	6.0–9.0
Growth temperature (°C)	4–37	10–37	10–30	10–37
NaCl tolerance (%, w/v)	0–0.5 %	0–2.5 %	0–2 %	0–1 %
Oxidase	+	−	−	+
β-Galactosidase	−	+	+	−
Hydrolysis of:				
DNA	−	+	−	−
Tween 20/80	+	+	+	−
Lipase (C14)	−	+	+	−
Trypsin	−	+	−	+
Naphthol-AS-BI-phosphopydrolase	+	−	+	+
α-Glucosidase	−	+	+	−
Nitrates to nitrites	−	−	+	+
Nitrates to nitrogen	+	+	−	−
Arginine dihydrolase	−	−	+	+
Urease	−	−	−	+
D-Glucose	+	+	−	+
N-acetyl-glucosamine	−	−	−	+
Maltose	−	+	+	−
Adipic acid	−	+	−	−
DNA G+C content (mol%)	67.7	61.7	70.6	67.1

Strain ZS60^T was incubated on R2A agar media for observation of cells and colony morphology. The morphology of strain ZS60^T was investigated by light microscope (Leica; ×1000), scanning electron microscope (S-3400N, Hitachi) and transmission electron microscope (Tecnai G2 Spirit BioTWIN) using cells from exponentially growing cultures. The Gram-staining was determined by using the classical Gram procedure [23] and was confirmed by using the KOH lysis test as described by Smibert and Krieg [24]. Strain ZS60^T formed pale yellow, circular, opaque and slightly convex colonies after incubation at 20 °C for 3 days on R2A agar. The cells of strain ZS60^T were aerobic, Gram-negative, non-motile, non-spore-forming and rod-shaped (about 0.3–0.5 µm wide and 1.1–2.3 µm long). Growth in R2A medium at different temperatures (−4, 4, 10, 20, 28, 37 and 42 °C) and various pH values (pH 4.0–12.0 at intervals of 1 pH units) was assessed after 10 days of incubation, and the growth of strains by measuring the OD_{600} every day. Salt tolerance was determined in R2A liquid medium supplemented with 1–5 % (w/v at intervals of 0.5 % unit) NaCl after 10 days of incubation (with shaking at 130 r.p.m.) at 24 °C, to inspect the growth by turbidity at OD_{600} by using a spectroscopic method (model EVO60, Thermo). Catalase and oxidase activity were determined by bubble production in 3 % (v/v) hydrogen peroxide solution and 1 % (w/v) *N-N-N′-N′*-tetramethyl *p*-phenylenediamine, respectively. Casein, starch, carboxymethyl cellulose and Tween 20/80 degradations were tested on R2A plates containing milk powder (5 %, w/v), starch (0.2 %, w/v), sodium carboxyl methyl cellulose (1 %, w/v) and Tween 20/80 (1 %, v/v), respectively. Strain ZS60^T grew well on nutrient agar, R2A, lysogeny broth and trypcase soy agar. The strain grew in the temperature range from 4 to 37 °C (optimum, 28 °C) and pH 6–10 (optimum, pH 7) and tolerated up to 0.5 % (w/v) NaCl (optimum, 0 %). Enzyme activities and carbon-source utilization were determined by using the API 20E, API 20NE, API CH50 and API ZYM test kits (bioMérieux) as per the manufacturer's instructions. The API ZYM tests were read after 6 h of incubation at 28 °C, the other API tests after 24 or 48 h at 28 °C. Differential phenotypic characteristics between strain ZS60^T and other related recognized *Lysobacter* species are summarized in Table 1.

For determination of quinones, cells grown exponentially in R2A were harvested by centrifugation, washed three times with distilled water and freeze-dried. Quinones were extracted and analysed by HPLC using the method of Collins *et al.* [25]. Total lipids extracted from strain ZS60^T grown in R2A liquid medium were examined by two-dimensional TLC with two developing solvents, chloroform/methanol/water (65 : 25 : 4, by vol.) and chloroform/methanol/acetic acid/water (80 : 12 : 18 : 5, by vol.). TLC plates were visualized with appropriate detection reagents (Fig S2) [26]. The cellular fatty acid profiles of strain ZS60^T, *L. daejeonensis* GH1-9^T, *L. caeni* BUT-8^T and *L. ruishenii* CTN-1^T were analysed by the Guangdong Culture Collection Centre by using gas chromatograph. All of the strains were cultured on R2A agar at 28 °C for 2 days, until the bacterial communities reached the late-exponential stage of growth. The cellular fatty acid profiles of strain ZS60^T and related *Lysobacter* type strains are shown in Table 2. The major cellular fatty acids in strain ZS60^T included iso-$C_{15:0}$ (23.4 %), iso-$C_{17:0}$ (17.2 %) and summed feature 9 (comprising iso-$C_{17:1}\omega9c$, 12.6 %). This fatty acid profile corresponded to other described species of the genus *Lysobacter* [27, 28]. However, some qualitative and quantitative differences in the fatty acid profiles were noted between ZS60^T and the reference strains. Compared with *L. daejeonensis* GH1-9^T, *L. caeni* BUT-8^T and *L. ruishenii* CTN-1^T, strain ZS60^T had higher levels of iso-$C_{11:0}$, iso-$C_{11:0}$ 3-OH and iso-$C_{17:0}$, and lower levels of $C_{16:0}$, iso-$C_{16:0}$ and summed feature 9. Q-8 was the major respiratory ubiquinone of strain ZS60^T. This is a characteristic feature of the genus *Lysobacte*r [29].

According to the data from 16S rRNA gene sequence comparisons and the low levels of ANI value and DNA–DNA relatedness, strain ZS60^T was assigned to the genus *Lysobacter*. The hybridization values were less than 70 %, which is the threshold value for the delineation of species [30]. Based on the morphological, biochemical and chemotaxonomic characteristics. We suggest that strain ZS60^T should be attributed to a novel species of the genus *Lysobacter*, for which we propose the name *Lysobacter psychrotolerans* sp. nov.

DESCRIPTION OF *LYSOBACTER PSYCHROTOLERANS* SP. NOV.

Lysobacter psychrotolerans (psy.chro.to′ le.rans. Gr. adj. *psychros* cold; L. pres. part. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* tolerating cold temperature).

Table 2. Cellular fatty acid content (%) of strain ZS60^T and other members of the genus *Lysobacter*

Strains: 1, ZS60^T; 2, *L. daejeonensis* GH1-9^T (data were obtained in this study); 3, *L. caeni* BUT-8^T (data were obtained in this study); 4, *L. ruishenii* CTN-1^T (data were obtained in this study). –,<1 % or not detected.

Fatty acid	1	2	3	4
iso-$C_{11:0}$	9.1	5.8	7.1	6.5
iso-$C_{11:0}$ 3-OH	9.6	6.6	7.9	4.3
iso-$C_{14:0}$	–	1.1	1.5	–
iso-$C_{15:0}$	23.4	22.3	21.3	25.6
iso-$C_{15:0}\omega9c$	4.5	–	–	1.6
anteiso-$C_{15:0}$	2.2		1.3	2.2
iso-$C_{15:1}$ F	–	1.6	1.5	–
$C_{16:0}$	1.6	6.1	4.4	5.6
iso-$C_{16:0}$	5.4	14.4	14.2	10.4
$C_{16:1}\omega7c$ alcohol	2.6	–	–	1.0
$C_{16:1}$ N alcohol	–	–	–	1.5
$C_{16:1}\omega11c$	1.18	–	–	2.0
iso-$C_{17:0}$	17.2	10.7	10.3	11.6
iso-$C_{17:1}\omega9c$	12.6	22.8	19.6	13.7
iso-$C_{17:1}\omega10c$	7.7	–	–	–
anteiso-$C_{17:0}$	–	–	1.0	–
Summed feature 3*	1.2	1.9	4.6	5.2

*Summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$

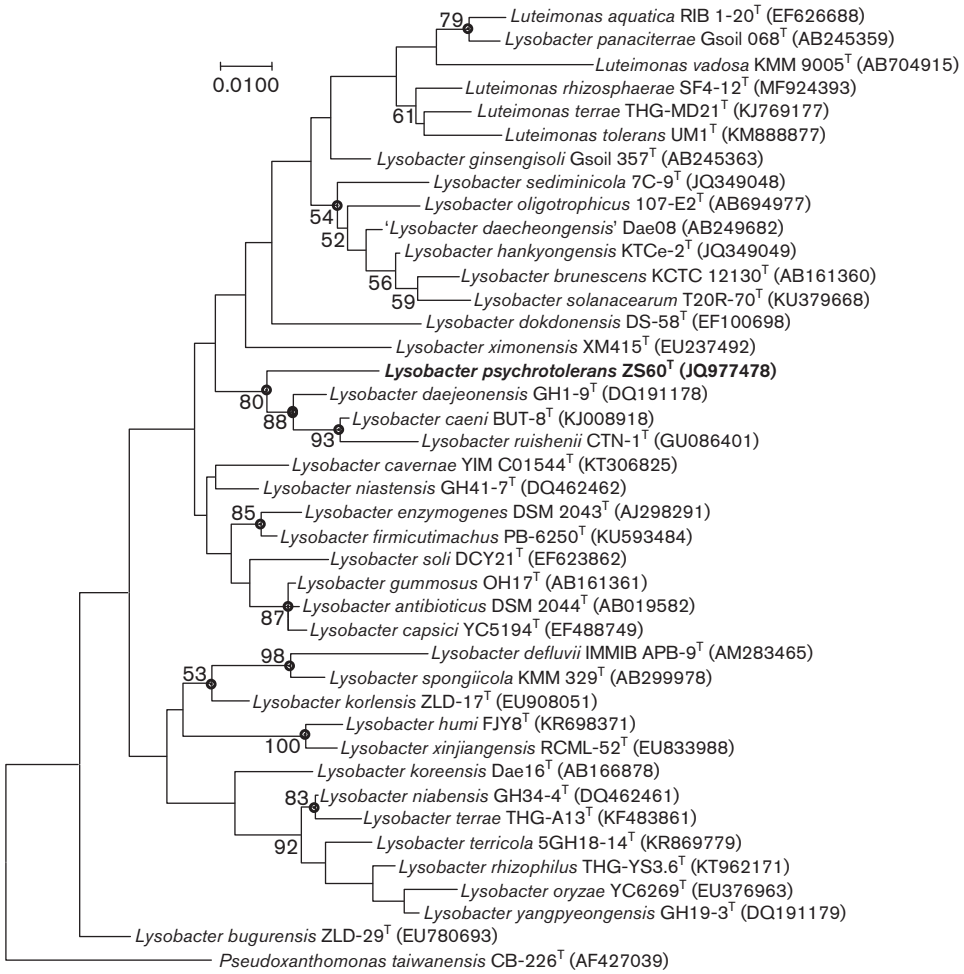

Fig. 1. Maximum-likelihood phylogenetic tree showing the taxonomic position of *Lysobacter psychrotolerans* ZS60^T based on the original described 16S rRNA gene sequence. Bootstrap values (%) are based on 1000 replicates and shown for branches with more than 50 % support. *Pseudoxanthomonas taiwanensis* CB-226^T was used as an outgroup. Circles indicate nodes that could be recovered in the phylogenetic trees generated with the maximum-parsimony and neighbour-joining methods. Bar, 0.01 substitutions per nucleotide position.

Cells are Gram-stain-negative, strictly aerobic, non-motile and rod-shaped. Cells are 0.3–0.5 µm wide and 1.1–2.3 µm in long. On R2A, it forms light yellow, circular, convex and wet colonies after 3 days. Grows at pH 6.0–10.0 (optimum, pH 7.0), 4–37 °C (optimum, 28 °C) and NaCl concentrations of 0–0.5 % (w/v; optimum, 0 %). Positive for catalase, oxidase and protease, but negative for H_2S production, DNA hydrolysis, starch hydrolysis and Tween 20/80 hydrolysis. API ZYM test showed positive results for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphopydrolase, esterase (C4) and esterase lipase (C8) activities. Negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, α-mannosidase, α-fucosidase, and *N*-acetyl-β-glucosaminidase activities. The API 20NE test showed negative results for indole and acetoin production. The Biolog test showed that D-glucose, maltose, trehalose, cellobiose, gentiobiose, turanose, lactose, melibiose, β-methyl-D-glucoside, D-fructose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-mannose, D-galactose, 3-methyl-glucose, D-fucose, L-fucose, D-sorbitol, D-mannitol, tween 20, Tween 80, myo-inositol, gelatin, glycyl-L-prolin, L-histidine, D-gluconic acid, D-glucuronic acid, D-malic acid, acetic acid, D-glucose-6-PO_4, D-fructose-6-PO_4, mucic acid and α-keto-glutaric acid are utilized as sole carbon and energy sources. However, dextrin, raffinose, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-neuraninic acid, L-rhamnose, inosine, D-arabitol, glycerol, D-aspartic, D-serine, L-alanine, L-aspartic acid, L-serine, pecine, D-gluconic acid, L-lactic acid, L-malic acid, bromo-succinic acid, α-keto-butyric acid, propionic acid and formic acid are not. Ubiquinone Q-8 is the predominant respiratory quinone, and the major polar lipids are diphosphatidylglycerol, phosphatidylelthanolamine, phosphatidylglycerol and aminolipid. The main fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$, iso-$C_{17:1}\omega 9c$ and

iso-$C_{11:0}$, and iso-$C_{11:0}$ 3-OH is the major 3-hydroxyl fatty acid.

The type strain, ZS60^T (=CGMCC 1.15509^T=NBRC 112614^T), was isolated from soil sampled at the Tianshan Mountains, China. The DNA G+C content of the type strain is 69.5 mol% (G+C content test) and 67.7 mol% (draft genome sequence). The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain ZS60^T is JQ977478. The whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession RIBS00000000.

Funding information
This study was supported by the National Natural Science Foundation of China (No. 31570488), China's Assistance Program to Developing Countries (No. KY201501008), the Open Project Program of Xinjiang Production and Construction Corps Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin (No. BRZD1601), and the Fundamental Research Funds for the Central Universities (No. lzujbky-2017-152).

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