Systematic and Applied Microbiology 42 (2019) 168–177

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: http://www.elsevier.com/locate/syapm

# Genetic diversity of glacier-inhabiting *Cryobacterium* bacteria in China and description of *Cryobacterium zongtaii* sp. nov. and *Arthrobacter glacialis* sp. nov.

Qing Liu, Hong-Can Liu, Yu-Guang Zhou *, Yu-Hua Xin *

*China General Microbiological Culture Collection Center (CGMCC), Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China*

ARTICLE INFO

*Article history:*
Received 31 March 2018
Received in revised form
20 September 2018
Accepted 8 October 2018

*Keywords:*
*Cryobacterium*
*Arthrobacter*
MLSA
Diversity
Glacier-inhabiting

ABSTRACT

*Cryobacterium* and *Arthrobacter* are members of *Actinobacteria*, and are often found in cold environments. In this study, 48 *Cryobacterium* strains, including 9 type strains and 39 new isolates collected from glaciers in China were subjected to multilocus sequence analysis (MLSA). Phylogenetic analysis revealed that *Cryobacterium* comprised four cold-adapted clusters. Also, 19 potential novel *Cryobacterium* species were found using 0.065 as the cut-off point of genetic distance between the concatenated gene sequences. Additionally, three *Cryobacterium* strains (TMN-42$^T$, TMN-39-1 and TMB1-8) and two *Arthrobacter* strains (HLT2-12-2$^T$, TMN-18) isolated from glaciers were subjected to taxonomic analysis. Based on 16S rRNA gene sequences, MLSA data and average nucleotide identity (ANI) values, they represented a novel *Cryobacterium* species and a novel *Arthrobacter* species. Specifically, strain TMN-42$^T$ was most closely related to the type strains of *Cryobacterium arcticum* and *Cryobacterium psychrotolerans* with 83.79% and 77.78% ANI values, respectively. The ANI values between strain HLT2-12-2$^T$ and its closely relatives *Arthrobacter psychrochitiniphilus* GP3$^T$ and *Arthrobacter alpinus* S6-3$^T$ were 76.66% and 77.94%, respectively. Therefore, we propose two novel species, *Cryobacterium zongtaii* sp. nov. (TMN-42$^T$ = CGMCC 1.9695$^T$ = NBRC 111591$^T$) and *Arthrobacter glacialis* sp. nov. (HLT2-12-2$^T$ = CGMCC 1.10025$^T$ = NBRC 113092$^T$).



## Introduction

Large numbers of glaciers located on the Qinghai-Tibet Plateau provide excellent living habitats for cold-adapted microorganisms. Recently, many research studies on the ecology and diversity of complex microbial communities on these glaciers have been conducted [21,22], and established a deeper understanding of the microbial distribution and phylogenetic diversity. One of the dominant groups on the glaciers is the phylum *Actinobacteria*, whose many strains belong to the genera *Cryobacterium* and *Arthrobacter* [6,23,27,35,43]. The two genera, within the order *Micrococcales*, are Gram-positive, non-spore forming, irregular rod-shaped, aerobic bacteria. During the course of the studies on bacterial community structure and biogeography on glaciers in China, we isolated a large number of psychrophilic and psychrotrophic bacterial strains, including numerous *Cryobacterium* and *Arthrobacter* strains [19,21].

The genus *Cryobacterium* was proposed by Suzuki et al. [38], whose first species was isolated from Antarctic soil by Inoue [13] and formerly named as "*Curtobacterium psychrophilum*" by Inoue and Komagata [14]. *Cryobacterium* strains are commonly found in cold environments, such as mountain glaciers [11,17,18,29], the Antarctic, Arctic and Siberian permafrost soils [5,33,38], Antarctic sandy intertidal sediments [45], surface seawater along the Victoria Land coast (Antarctica) [24] and glacier cryoconite holes of the High Arctic [36]. *Cryobacterium* was considered to be a rare taxon in the 454-pyrosequencing data set from surface samples of six glaciers in China [21]. However, we isolated many *Cryobacterium* strains, and four of them have been validly described as novel species [17,18,20]. To date, the genus *Cryobacterium* contains nine recognized species. The almost identical 16S rRNA gene sequences often corresponded to a very low DNA–DNA hybridization (DDH) value between *Cryobacterium* species [18]. Accordingly, the poor discrimination of the 16S rRNA gene sequence leads to difficulty in the identification of very closely related *Cryobacterium* species. Moreover, the diversity of *Cryobacterium* species on the glaciers may be grossly underestimated using the 16S rRNA gene.

* Corresponding authors at: No. 1 West Beichen, Chaoyang District, Beijing 100101, PR China.
*E-mail addresses:* zhouyg@im.ac.cn (Y.-G. Zhou), xinyh@im.ac.cn (Y.-H. Xin).

https://doi.org/10.1016/j.syapm.2018.10.005
0723-2020/

**Table 1**
PCR primers used to amplify *Cryobacterium* genes *atpD*, *dnaK*, *recA*, *rpoB*, *secY* and *ychF*.

| Locus | Primer name | Primer sequence (5′ – 3′) | Amplicon size (bp) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|---|
| *atpD* | atpD743F<br>atpD1247R | ACAACATCTTCCGCTTCAC<br>AACTTCTTSGCCATGTASG | 504 | 52 | This study |
| *dnaK* | dnaK190F<br>dnaK889R | GAGGTSGGCAAGGACGACGAST<br>TYGCVACGGACGGCTGGTT | 699 | 59 | This study |
| *recA* | recA63F<br>recA690R | CGGMAARGGMTCGGTCAT<br>SGTYTCRATRCGGCGGATGT | 627 | 58 | This study |
| | recA56F[a]<br>recA689R[a] | GBCARTTCGGMAARGGMT<br>GTYTCRATRCGGCGGATGT | 633 | 52 | This study |
| *rpoB* | rpoB2473F<br>rpoB3303R | GGHAAGGTSACSCCNAAGGG<br>GAANCGCTGDCCRCCGAACTG | 754 | 60 | [1] |
| *secY* | secY238F<br>secY1109R | GGBRTBATGCCSTACATYAC<br>AANCCRCCRWACTKCTTCAT | 787 | 52 | [1] |
| *ychF* | ychF208F<br>ychF983R | TTYGTBGAYATCGCVGG<br>ACGAYYTCVGCYTTGATGAA | 703 | 52 | [1] |

[a] Alternative primer for *recA*.

*Arthrobacter* has been considered to be distributed worldwide, and has been isolated from various environments, such as soil, sludge, sewage, lake and glacier [9]. Species of *Arthrobacter* display significant heterogeneity regarding phenotypic characteristic, e.g. the composition of quinones, peptidoglycans, and polar lipids [8]. Based on the 16S rRNA gene phylogeny and chemotaxonomic characteristics, the genus *Arthrobacter* was revised into seven genera [8,34]. On glaciers in China, two new *Arthrobacter* clusters were discovered [19]. In this study, two new glacier-inhabiting strains were analyzed and found to belong to the '*Arthrobacter psychrolactophilus* group' [8,12,25,42,47].

Multilocus sequence analysis (MLSA) has been shown to be a powerful method for the taxonomic study of certain bacterial genera [26,28,31,37,40] and the study of bacterial diversity [2,3,7,10]. In the present study, we conducted a MLSA of 48 *Cryobacterium* strains, including 9 type strains and 39 new isolates collected from glaciers in China, to improve the taxonomy and identification of this genus and reveal the genetic diversity of glacier-inhabiting *Cryobacterium* strains. In addition, a novel psychrotolerant species, namely *Cryobacterium zongtaii* sp. nov., isolated from the Touming Mengke glacier and a novel psychrotolerant species, namely *Arthrobacter glacialis* sp. nov., isolated from the Touming Mengke glacier and Hailuogou glacier are described and proposed based on the phenotypic, genotypic and phylogenetic characterization.

## Materials and methods

### *Bacterial strains and culture conditions*

Type strains of *Cryobacterium* were obtained from the China General Microbiological Culture Collection Center (CGMCC). A total of 39 *Cryobacterium* isolates (Table S1), which were previously collected [21] from Xinjiang No. 1 glacier (NO1), Touming Mengke glacier (TM), Hailuogou glacier (HLG), and Midui glacier (MD) in China, were selected for analysis. All these strains were routinely incubated on peptone, yeast extract and glucose (PYG) medium [16] at 14 °C except for *Cryobacterium mesophilum* CGMCC 1.10440$^T$, which was incubated at 28 °C.

Strains HLT2-12-2$^T$ and TMN-18 were isolated from top soil samples from the Hailuogou glacier and Touming Mengke glacier, respectively [21]. The two strains were subjected to a phylogenetic analysis and classified into the genus *Arthrobacter*. The three closely related type strains of recognized species in '*A. psychrolactophilus* group' (*Arthrobacter psychrochitiniphilus* CGMCC 1.6355$^T$, *Arthrobacter psychrolactophilus* CGMCC 1.9101$^T$ and *Arthrobacter alpinus* CGMCC 1.8950$^T$) were used as reference. The HLT2-12-2$^T$, TMN-18 and reference strains were routinely incubated on tryptic soy agar (TSA) medium (BD Difco; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 25 °C.

### *DNA extraction, amplification, and sequencing*

The Genomic DNA Rapid Isolation Kit for Bacterial Cell (BioDev-Tech, Beijing, China) was used to extract genomic DNA from the various bacterial strains. For the MLSA of the *Cryobacterium* strains, the genome sequences of *Cryobacterium* sp. MLB-32 (GCF_000738085), *Cryobacterium roopkundense* RuGl7$^T$ (NZ_JPXF01000001), *Leifsonia xyli* subsp. *xyli* CTCB07 (AE016822) and *Clavibacter michiganensis* subsp. *sepedonicus* ATCC 33113$^T$ (AM849034) were used to design primers for the housekeeping genes: *atpD* (ATP synthase β subunit), *dnaK* (chaperone protein DnaK) and *recA* (homologous recombination factor). Three housekeeping genes: *rpoB* (DNA-directed RNA polymerase β subunit), *secY* (protein translocase subunit) and *ychF* (GTP-binding and nucleic acid-binding protein) were amplified and sequenced as described by Adekambi et al. [1]. Sequencing was performed using a PRISM 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) at SinoGenoMax Co. (Beijing).

### *Nucleotide polymorphism and phylogenetic analysis*

The 16S rRNA gene sequences of the test strains were retrieved from the GenBank database using the accession numbers and compared with those of recognized type strains using the EzBioCloud database [44]. Six housekeeping genes (*rpoB*, *recA*, *atpD*, *fusA*, *secY* and *tuf*) sequences of the two *Arthrobacter* strains were obtained from the genome sequences and the related sequences were from Liu et al. [19] or GenBank. The CLUSTAL W program [41] was used to perform multiple sequence alignment. Sequences were concatenated using the BioSuite program (https://github.com/dongzhang0725/BioSuite). The locus characteristics, including number of segregating sites, nucleotide diversity (Pi) and sequence similarities for *Cryobacterium* strains, were determined by MEGA V5.0. [39]. Phylogenetic trees were constructed by MEGA V5.0 using the neighbor-joining (NJ) method with the Kimura two-parameter (K2P) model and maximum likelihood (ML) method with the GTR+G+I model. The tree topologies were evaluated using bootstrap analysis based on 1000 reiterations.

### *Phenotypic and genotypic characterization of novel species*

Three *Cryobacterium* strains, namely TMN-42$^T$, TMN-39-1 and TMB1-8, and two *Arthrobacter* strains, namely HLT2-12-2$^T$ and

**Table 2**
Gene characteristics of 48 *Cryobacterium* strains.

| | Length (nt) | *S* | $P_s$ (%) | K2P distance (range) | $\pi$ | Tajima'D |
|---|---|---|---|---|---|---|
| 16S rRNA | 1358 | 98 | 7.28 | 0–0.041 | 0.0163 | −0.0196 |
| *atpD* | 438 | 135 | 30.82 | 0–0.206 | 0.0794 | 0.5163 |
| *dnaK* | 627 | 190 | 30.30 | 0–0.167 | 0.0624 | −0.3121 |
| *recA* | 507 | 171 | 33.73 | 0–0.190 | 0.1092 | 1.5821 |
| *rpoB* | 681 | 207 | 30.40 | 0–0.184 | 0.0924 | 1.2650 |
| *secY* | 744 | 343 | 46.10 | 0–0.308 | 0.1482 | 1.5586 |
| *ychF* | 651 | 292 | 44.85 | 0–0.302 | 0.1288 | 0.9986 |
| MLSA[a] | 3648 | 1338 | 36.68 | 0.001–0.215 | 0.1059 | 1.0340 |

*S*, number of segregating sites; $P_s$, percentage of segregating sites; $\pi$, nucleotide diversity.
[a] *atpD-dnaK-recA-rpoB-secY-ychF* concatenated gene sequences.

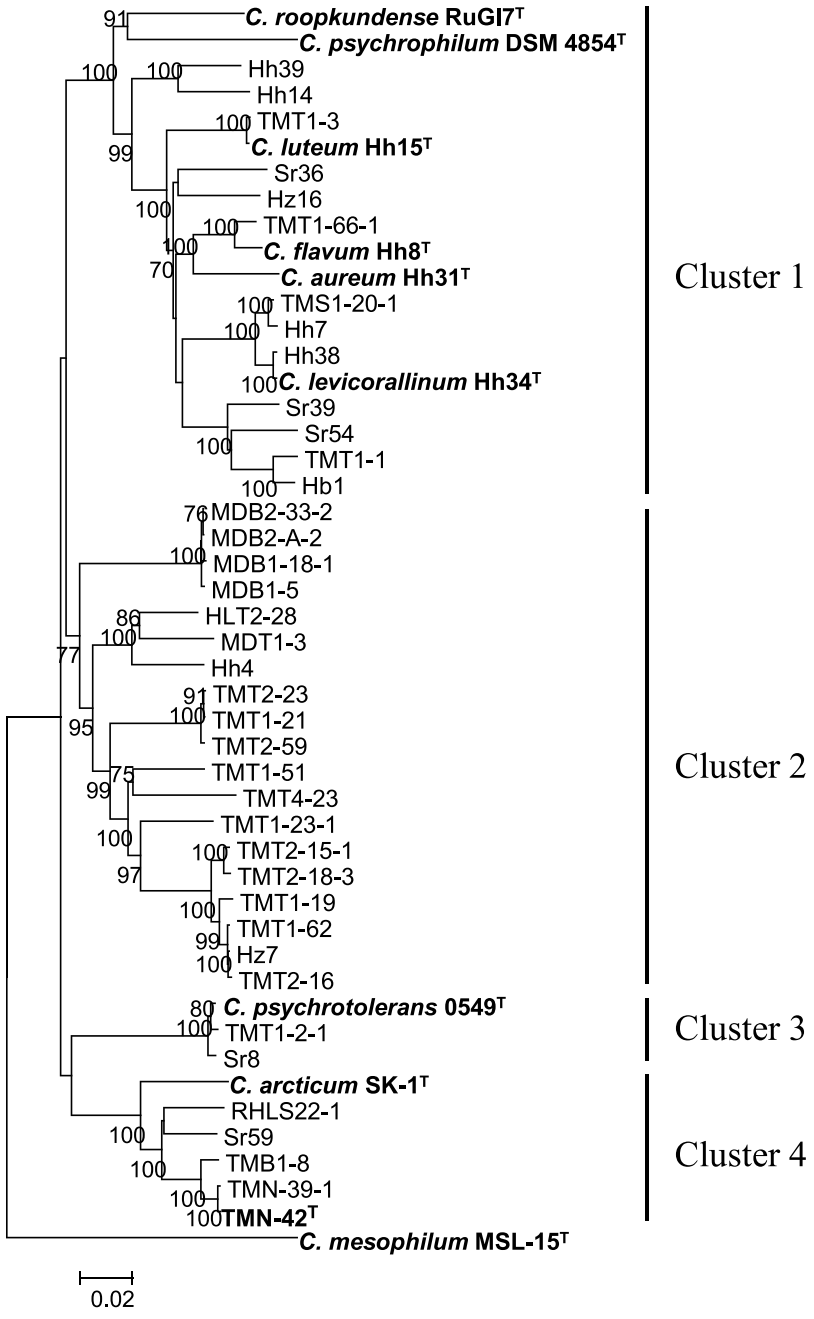


**Fig. 1.** Phylogenetic tree based on *atpD*, *dnaK*, *recA*, *rpoB*, *secY* and *ychF* gene sequences (3648 nt), using the NJ method. Bootstrap values (≥70%) based on 1000 replicates are shown at branch nodes. Bar: two nucleotide substitutions per 100 positions.

TMN-18, were subjected to a polyphasic taxonomic analysis. To determine the relatedness of the novel strains with the closest related species, the draft genomes of the five glacier-inhabiting strains and the reference strains *Cryobacterium arcticum* SK-1$^T$, *A. psychrochitiniphilus* CGMCC 1.6355$^T$, *A. psychrolactophilus* CGMCC 1.9101$^T$, *Arthrobacter stackebrandtii* CGMCC 1.9110$^T$ and *Arthrobacter livingstonensis* CGMCC 1.15658$^T$ were sequenced using the Illumina Hiseq 4,000 platform (Illumina, San Diego, CA, USA). Short reads were assembled using SPAdes 3.11 [4]. The genome sequences of the reference strains *Cryobacterium psychrotolerans* CGMCC 1.5382$^T$ and *A. alpinus* S6-3$^T$ were retrieved from GenBank using the accession numbers NZ_FNFU01000001 and GCF_900105965, respectively. The average nucleotide identity (ANI) based on the BLASTN algorithm was calculated using the JSpecies software tool [30].

For the analysis of respiratory quinones, polar lipids, peptidoglycan and cell-wall sugars, cells of the *Cryobacterium* strain TMN-42$^T$ were harvested after culturing in PYG broth at 20 °C for 4 days, and cells of *Arthrobacter* strain HLT2-12-2$^T$ were harvested after cultured in TSB broth at 25 °C for 4 days. For analysis of cellular fatty acid composition, the biomasses of the test *Cryobacterium* and *Arthrobacter* strains were harvested at the end of the exponential growth phase from PYG medium after incubation at 20 °C and TSA medium after incubation at 25 °C, respectively. Respiratory quinones, polar lipids, cell-wall peptidoglycan amino acids, whole-cell sugars and cellular fatty acids were extracted and analyzed using previously described methods [15,32]. After purification and hydrolysis, the peptidoglycan hydrolysates and standard amino acids were examined using a high-speed amino acid analyzer L-8900 (Hitachi Ltd., Tokyo, Japan). The partial hydrolysates were analyzed by QTRAP 6500 LC-MS/MS System. The tests of carbon source utilization were performed using the Biolog GEN III Microstation (Biolog, Hayward, CA, USA). Other physiological properties were tested as previously described [17,18] or using the API 20NE, API 20E, API ZYM and API 50CH strips (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. Growth was examined at different temperature (4, 10, 14, 18, 20, 22, 24, 25, 26 and 30 °C), different NaCl concentration (1.0–6.0% (w/v), at the interval of 0.5%) and different pH (4.0–12.0, at the interval of 1.0).

### *Nucleotide sequence accession numbers*

GenBank accession numbers for *atpD*, *dnaK*, *recA*, *rpoB*, *secY* and *ychF* gene sequences of *Cryobacterium* strains determined in this study are MF741358-MF741645 (Table S1). The GenBank accession numbers for the 16S rRNA gene sequence of type strains TMN-42$^T$ and HLT2-12-2$^T$ are JX949938 and JX949500, respectively. The genome sequences have been deposited at GenBank under accession numbers described in Table S2.

## Results and discussion

### *Diversity of the* Cryobacterium *on the glaciers based on MLSA*

The sequences of six housekeeping genes (*atpD*, *dnaK*, *recA*, *rpoB*, *secY* and *ychF*) were successfully determined for all *Cryobacterium* strains using the primers listed in Table 1. The nucleotide polymorphism statistics of the 48 *Cryobacterium* strains are summarized in Table 2. There were no significant Tajima's D values, indicating a neutral evolution pattern for the housekeeping genes. The six housekeeping genes were obviously more informative than the 16S rRNA gene, specifically, the *secY* and *ychF* gene, showed the highest degree of variation. The pairwise K2P distance ranges of the *secY* and *ychF* genes were 0–0.308 and 0–0.302, respectively.

**Table 3**
Sequence similarities of 16S rRNA genes (upper right "triangle"), MLSA data (upper right "triangle" with []) and ANI values (lower left "triangle") between strains TMN-42$^T$, TMN-39-1, TMB1-8 and related species.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | | 100 [99.8] | 99.80 [98.6] | 99.27 [93.4] | 98.13 [89.0] |
| 2 | 99.06 | | 99.80 [98.7] | 99.27 [93.4] | 98.13 [88.9] |
| 3 | 96.14 | 96.06 | | 99.49 [93.8] | 98.06 [89.2] |
| 4 | 83.79 | 83.84 | 83.82 | | |
| 5 | 77.78 | 77.81 | 77.84 | 77.80 | |

Strains: (1) TMN-42$^T$; (2) TMN-39-1; (3) TMB1-8; (4) *C. arcticum* SK-1$^T$; (5) *C. psychrotolerans* 0549$^T$.

**Table 4**
Differential characteristics of *Cryobacterium zongtaii* and its closest related species.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Acids produced from: | | | | | |
| Glycerol | + | + | + | – | – |
| L-Rhamnose | – | – | – | + | – |
| D-Cellobiose | + | + | + | + | – |
| D-Raffinose | – | – | – | + | – |
| D-Melibiose | – | – | – | + | – |
| L-Fucose | – | – | – | – | + |
| Assimilation of: | | | | | |
| D-Cellobiose | + | + | + | + | – |
| Gentiobiose | + | + | + | + | – |
| D-Turanose | + | + | + | + | – |
| Stachyose | – | – | – | + | – |
| D-Taffinose | + | – | + | + | – |
| α-D-lactose | + | + | + | + | – |
| D-Melibiose | – | – | + | + | – |
| β-methyl-D-glucoside | + | + | + | + | – |
| D-Salicin | + | + | + | + | – |
| *N*-Acetyl-β-D-mannosamine | + | + | – | – | – |
| *N*-Acetyl-D-galactosamine | – | – | – | – | + |
| *N*-Acetyl neuraminic acid | + | + | + | – | – |
| L-Fucose | – | – | – | – | + |
| L-Rhamnose | – | – | – | + | – |
| D-Sorbitol | – | – | + | w | – |
| D-Mannitol | + | + | + | + | – |
| Gelatin | – | – | – | + | + |
| Glycyl-L-proline | + | + | – | – | – |
| L-Alanine | – | – | – | – | + |
| L-Aspartic acid | + | + | + | – | + |
| L-Glutamic acid | + | + | + | – | + |
| L-Histidine | + | – | + | – | – |
| L-Pyroglutamic acid | – | – | – | – | + |
| Pectin | + | + | + | + | – |
| D-Galacturonic acid | + | + | – | – | – |
| L-Galactonic acid lactone | + | + | – | + | – |
| D-Gluconic acid | + | + | + | + | – |
| *p*-Hydroxy-phenylacetic acid | – | – | – | – | + |
| Methyl pyruvate | + | + | + | + | – |
| L-Lactic acid | – | – | – | + | + |
| L-Malic acid | + | + | + | – | + |
| Bromo-succinic acid | + | + | + | – | + |
| γ-amino butryric acid | – | – | – | – | + |
| β-hydroxy-D,L-butyric acid | – | – | – | + | + |
| Formic acid | + | + | – | – | w |

Strains: (1) TMN-42$^T$; (2) TMN-39-1; (3) TMB1-8; (4) *C. arcticum* SK-1$^T$; (5) *C. psychrotolerans* 0549$^T$. (+) Positive; (–) negative; (w) weakly positive.

The pairwise K2P distance ranges of the *atpD*, *dnaK*, *recA*, *rpoB* and concatenated genes were 0–0.206, 0–0.167, 0–0.190, 0–0.184 and 0.001–0.215, respectively. Therefore, the classification and identification of *Cryobacterium* using the housekeeping genes were reliable and informative. The closest genetic distance of the concatenated gene sequences between the 9 recognized species was 0.065. When the 0.065 distance of the concatenated gene sequences was used as the threshold to differentiate two *Cryobacterium* species, 7 isolates

**Table 5**
Protologue for *Cryobacterium zongtaii* sp. nov.

| | |
|---|---|
| Taxonumber | TA00352 |
| Species name | *Cryobacterium zongtaii* |
| Genus name | *Cryobacterium* |
| Species status | sp. nov. |
| Species etymology | zong. ta'i.i. N.L. gen. n. *zongtaii* of Zong-Tai, named in honour of the Chinese glaciologist Zong-Tai Wang for his contributions to the name of Touming Mengke glacier |
| Designation of the type strain | TMN-42 |
| Strain collection numbers | CGMCC 1.9695, NBRC 111591 |
| 16S rRNA gene accession number | JX949938 |
| Alternative housekeeping genes:gene [Accession number] | *atpD* [MF741390], *dnaK* [MF741438], *recA* [MF741486], *rpoB* [MF741534], sec*Y* [MF741582], *ychF* [MF741630] |
| Genome accession number | PPXD00000000 |
| Genome status | Draft |
| Genome size | 3.86 Mb |
| GC mol% | 67.6 |
| Country of origin | China |
| Region of origin | Gansu province |
| Date of isolation | 01 December 2009 |
| Source of isolation | Glacier |
| Sampling date | 10 October 2009 |
| Geographic location | Touming Mengke glacier |
| Number of strains in study | 3 |
| Source of isolation of non-type strains | Glacier |
| Grouth medium, incubation conditions | Medium [W/V; bacto peptone (Difco) 0.5%, yeast extract 0.02%, glucose 0.5%, beef extract 0.3%, NaCl 0.05%, $MgSO_4{\cdot}7H_2O$ 0.15%], temperature [20 °C], pH [7.0] |
| Conditions of preservation | Freeze dried, 10% (v/v) glycerol at −180 °C |
| Gram stain | POSITIVE |
| Cell shape | Rod |
| Cell size (length or diameter) | 1.8–2.6 μm in long and 0.8–0.9 μm in wide |
| Motility | Motile |
| If motile | Flagellar |
| If flagellated | Monotrichous |
| Sporulation resting cells | None |
| Colony morphology | Matt light yellow, convex, round (0.5–1.0 mm in diameter) |
| Temperature range | 4–24 °C |
| Lowest temperature for growth | 4 °C |
| Highest temperature for growth | 24 °C |
| Temperature optimum | 20 °C |
| Lowest pH for growth | 6 |
| Highest pH for growth | 10 |
| pH optimum | 7 |
| pH category | Neutrophile |
| Lowest NaCl concentration for growth | 0 |
| Highest NaCl concentration for growth | 3 |
| Salinity optimum | 0.5–2 |
| Relationship to $O_2$ | Aerobe |
| Positive tests with BIOLOG | Dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, D-taffinose, α-D-lactose, β-methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, inosine, D-mannitol, glycerol, glycyl-L-proline, L-aspartic acid, L-glutamic acid, L-histidine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, methyl pyruvate, L-malic acid, bromo-succinic acid, Tween 40, α-hydroxy butyric acid, propionic acid, acetic acid, formic acid, 1% sodium lactate, guanidine HCl (weakly), nalidixic acid, potassium tellurite (weakly), aztreonam and sodium bromate (weakly). |
| Negative tests with BIOLOG | Stachyose, D-melibiose, *N*-acetyl-D-galactosamine, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, D-sorbitol, D-arabitol, myo-inositol, D-glucose- 6-$PO_4$, D-fructose- 6-$PO_4$, D-aspartic acid, D-serine, gelatin, L-alanine, L-pyroglutamic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxy-phenylacetic acid, D-lactic acid methyl ester, L-lactic acid, citric acid, α-keto-glutaric acid, D-malic acid, γ-amino butryric acid, β-hydroxy-D, L-butyric acid, α-keto-butyric acid, acetoacetic acid, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, lithium chloride and sodium butyrate. |
| Positive tests with API | ESC, PNPG, ONPG, VP, MAN, SAC, AMY, ARA |
| Negative tests with API | NO3, TRP, GLU, ADH, URE, GEL, LDC, ODC, CIT, H2S, URE, TDA, IND |
| Acid formation from carbohydrates (all positive) | Glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, mannitol, amygdaline, esculine, D-cellobiose, D-maltose, D-lactose, D-saccharose and D-turanose |
| Acid formation for carbohydrates (negative) | Erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-Glucopyranoside, *N*-acetylglucosamine, arbutine, salicine, D-melibiose, D-trehalose, inuline, D-melezitose, D-raffinose, starch, glycogene, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-cetogluconate, potassium 5-cetogluconate |
| Commercial kits used | BIOLOG GEN III, API 20E, API 20 NE, API 50CH |
| Energy metabolism | Chemoorganotroph |
| Oxidase | Negative |
| Catalase | Positive |
| Negative tests | Tween 20, Tween 60, Tween 80, casein and starch |
| Quinone type | Predominantly MK-10, followed by MK-11 and MK-9 |
| Major fatty acids | $C_{15:1}$ iso G (9.0%), $C_{15:1}$ anteiso A (4.8%), $C_{17:1}$ iso ω5c (10.4%), $C_{16:0}$ iso (20.3%), $C_{15:0}$ anteiso (35.0%), $C_{17:0}$ anteiso (12.2%), $C_{16:0}$ (5.2%) |
| Peptidoglycan type | 2,4-Diaminobutyric acid is the major cell-wall diamino acid |
| Phospolipid pattern or diagnostiv phospolipid | Diphosphatidylglycerol, phosphatidylglycerol, two unidentified phospholipids and three unidentified lipids. |
| Glycolipids | Dimannosylglyceride |
| Biosafety level | 1 |

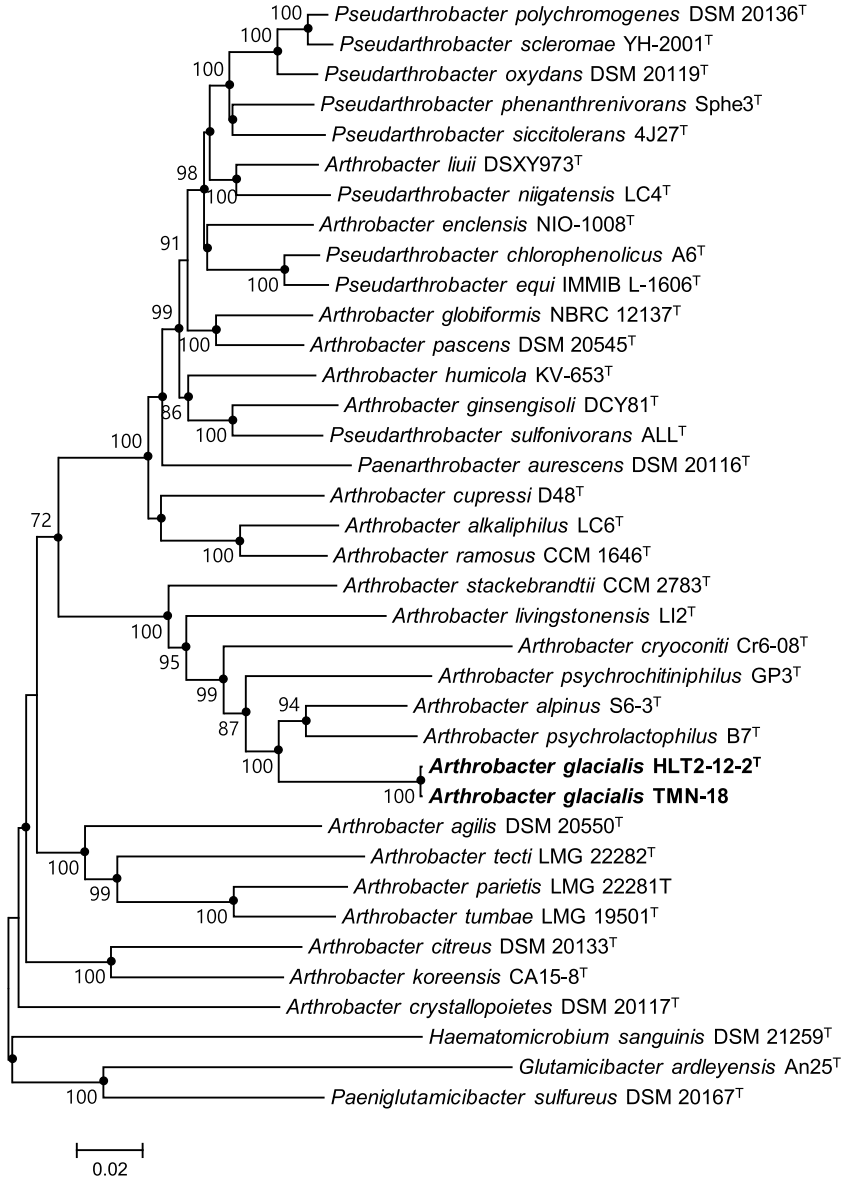


**Fig. 2.** Neighbor-joining tree of HLT2-12-2$^T$ and related taxa based on MLSA data (*tuf-secY-rpoB-recA-fusA-atpD*, 3762 nt). Filled circles indicate that the corresponding branches were recovered in the NJ and ML trees. Bootstrap values (≥70%) based on 1000 replicates are shown at branch nodes. Bar, 0.02 substitutions per nucleotide positions. The data of *A. enclensis* NIO-1008$^T$ (GCF_001457025) and *Haematomicrobium sanguinis* DSM 21259$^T$ (GCF_000688395) were extracted from the genome sequences in EzBioCloud.

were classified into the recognized species and 32 isolates were classified into 19 novel species.

Although certain strains shared identical 16S rRNA gene sequences, large diversity of *Cryobacterium* were discovered on the glaciers (Fig. S1). Moreover, the housekeeping genes distinguished the closely related strains and showed higher diversity than the 16S rRNA gene (Fig. 1). The ML phylogenetic tree based on the MLSA data (*atpD*, *dnaK*, *recA*, *rpoB*, *secY* and *ychF* gene) showed exactly the same topology as that of the NJ tree. The MLSA tree was divided into two large clusters (clusters 1 and 2), two small clusters (clusters 3 and 4) and *C. mesophilum* MSL-15$^T$. Cluster 1, with 100% bootstrap value, included 13 new *Cryobacterium* isolates and 6 recognized psychrophilic species: *C. roopkundense*, *Cryobacterium psychrophilum*, *Cryobacterium luteum*, *Cryobacterium flavum*, *Cryobacterium levicorallinum* and *Cryobacterium aureum*. Cluster 2, with 71% bootstrap value, included 19 new strains. Cluster 3 included *C. psychrotolerans* and two strains TMT1-2-1 and Sr8. Cluster 4 included *C. arcticum* and 5 new strains with 100% bootstrap value. The discovery of four clusters and 19 potential new species greatly expands our knowledge about this taxon, and improves the understanding of the genetic diversity of the genus *Cryobacterium* and its ecological distribution on the glaciers.

### *Phenotypic and genotypic characterization of a novel* Cryobacterium *species*

To clarify the taxonomic status of certain strains, polyphasic taxonomic analyses were performed in this study. The almost complete 16S rRNA gene sequences of strains TMN-42$^T$ (1466 bp), TMN-39-1 (1463 bp) and TMB1-8 (1463 bp) were determined. Strain TMN-42$^T$ showed 100% 16S rRNA gene sequence similarity with TMN-39-1, 99.8% sequence similarity with TMB1-8 and was most closely related to the type strains of *C. arcticum* and *C. psychrotolerans* with 99.27% and 98.13% sequence similarity, respectively

(Table 3). The similarities of the *atpD-dnaK-recA-rpoB-secY-ychF* concatenated gene sequences between strain TMN-42$^T$ and the two closely related type strains were 93.4% and 89.0%, respectively, (Table 3), which are in the range of those found for recognized species (78.6–93.5%). High similarities (>98.5%) of the concatenated gene sequences were found between strains TMN-42$^T$, TMN-39-1 and TMB1-8. Therefore, the three new strains represented a putative novel species of the genus *Cryobacterium*.

To test the above hypothesis, the genomes were sequenced for ANI calculation. The genome size of the *Cryobacterium* strain TMN-42$^T$ was about 3.86 Mb. The DNA G + C content of strain TMN-42$^T$ was 67.6 mol%, as determined from the draft genome sequence. ANI values higher than 96% were found among strains TMN-42$^T$, TMN-39-1 and TMB1-8, while ANI values lower than 95% were found between the three new strains and their closest related species (Table 3). Thus, they belong to a novel species of the genus *Cryobacterium*.

The cellular fatty acid compositions of the three *Cryobacterium* novel strains are displayed in Table S3, together with the results of the reference strains, which were analyzed under the same condition. The predominant fatty acids in strains TMN-42$^T$, TMN-39-1 and TMB1-8 were anteiso-$C_{15:0}$, anteiso A-$C_{15:1}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$ and iso-$C_{17:1}\omega5c$, which showed no significant difference compared with the reference strains. The menaquinone composition of strain TMN-42$^T$ was predominantly MK-10, followed by MK-11 and MK-9, and was similar to its closest relative *C. arcticum* SK-1$^T$ [5]. Strain TMN-42$^T$ contained 2, 4-diaminobutyric acid, which is the major cell-wall diamino acid of the genus *Cryobacterium*. Rhamnose, galactose, glucose and xylose were the sugar components of the whole-cell. Its polar lipids were diphosphatidylglycerol, phosphatidylglycerol, dimannosylglyceride, two unidentified phospholipids and three unidentified lipids (Fig. S2). The unidentified phospholipids and lipids found in strain TMN-42$^T$ were absent in strains *C. arcticum* SK-1$^T$ and *C. psychrotolerans* 0549$^T$ [5,46].

Cells of strain TMN-42$^T$ were Gram-stain positive, aerobic, rod shaped and 1.8–2.6 × 0.8–0.9 μm in size. The strain was motile and a single flagellum was observed (Fig. S3). The phenotypic differences between strains TMN-42$^T$, TMN-39-1, TMB1-8 and the reference strains are summarized in Table 4. The phenotypic characteristics of the three strains provide more evidence for their classification as a novel species of *Cryobacterium*. The novel strains can be differentiated from their closest related species by the 16S rRNA and housekeeping gene sequences, ANI values and phenotypic characteristics. Therefore, they should be classified as a novel species of the genus *Cryobacterium*, for which the name *C. zongtaii* sp. nov. is proposed. Strain TMN-42$^T$ (=CGMCC 1.9695$^T$ = NBRC 111591$^T$) is the type strain. The full description of the new taxon is shown in Table 5 as obtained from the Digital Protologue database website (http://imedea.uibcsic.es/dprotologue/) in which the new species was registered under reference TA00352.

**Table 6**
Phenotypic characteristics that differentiate HLT2-12-2$^T$ from the reference strains.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Nitrate reduction | + | + | – | – | – |
| Assimilation of: | | | | | |
| Stachyose | + | + | – | + | + |
| L-Fucose | + | + | – | + | + |
| L-Rhamnose | – | – | – | + | + |
| Inosine | + | + | – | w | + |
| D-Sorbitol | + | – | + | + | + |
| D-Arabitol | – | – | + | + | + |
| Myo-inositol | – | – | – | + | – |
| D-Glucose-6-$PO_4$ | – | – | – | – | + |
| D-Fructose-6-$PO_4$ | – | – | – | – | + |
| D-Serine | + | + | – | – | – |
| Gelatin | – | – | – | – | + |
| L-Alanine | + | + | – | w | – |
| L-Aspartic acid | + | + | + | – | + |
| L-Pyroglutamic acid | + | + | + | – | – |
| Pectin | – | – | w | – | – |
| D-Glucuronic acid | + | + | – | w | + |
| Glucuronamide | + | w | w | – | + |
| *p*-Hydroxy-phenylacetic acid | + | + | – | – | – |
| Methyl pyruvate | + | w | + | – | – |
| D-Malic acid | – | – | – | w | + |
| L-Malic acid | + | + | + | – | + |
| Bromo-succinic acid | + | + | + | – | + |
| Tween 40 | – | w | + | – | – |
| Y-amino butryric acid | + | + | + | + | – |
| α-hydroxy butyric acid | + | + | + | – | + |
| β-hydroxy-D,L-butyric acid | – | – | w | + | + |
| α-keto-butyric acid | + | + | + | – | + |
| Acetoacetic acid | + | + | w | – | – |
| Acetic acid | + | + | + | – | + |
| Formic acid | – | – | + | – | – |

Strains: (1) HLT2-12-2$^T$; (2) TMN-18; (3) *A. psychrochitiniphilus* CGMCC 1.6355$^T$; (4) *A. alpinus* CGMCC 1.8950$^T$; (5) *A. psychrolactophilus* CGMCC 1.9101$^T$. (+) Positive; (–) negative; (w) weakly positive.

## *Phenotypic and genotypic characterization of a novel* Arthrobacter *species*

The almost complete 16S rRNA gene sequences of the *Arthrobacter* strains HLT2-12-2$^T$ (1525 bp) and TMN-18 (1525 bp) were obtained. Pairwise similarity analysis showed that the two strains shared 100% sequence similarity and therefore only strain HLT2-12-2$^T$ was included in the 16S rRNA gene phylogenetic analysis. Sequences comparison revealed that strain HLT2-12-2$^T$ is most similar to species currently classified in the genus *Arthrobacter*. The highest sequence similarity was found with *A. psychrochitiniphilus* GP3$^T$ (99.17%), followed by *A. alpinus* S6-3$^T$ (98.63%) and *A. psychrolactophilus* B7$^T$ (98.30%). In the 16S rRNA gene phylogenetic tree (Fig. S4), strain HLT2-12-2$^T$ clustered into the '*A. psychrolactophilus* group' (100% bootstrap value) and formed a small cluster with the closest related species *A. psychrochitiniphilus*, *A. alpinus* and *A. psychrolactophilus*.

To clarify the phylogenetic affiliation of the novel isolates, MLSA using the concatenated sequences of the *tuf*, *secY*, *rpoB*, *recA*, *fusA* and *atpD* genes was also conducted. In the phylogenetic tree (Fig. 2), strains HLT2-12-2$^T$ and TMN-18 formed an independent branch and clustered with *A. psychrolactophilus* B7$^T$, *A. alpinus* S6-3$^T$ and *A. psychrochitiniphilus* GP3$^T$ (87% bootstrap value). Strains HLT2-12-2$^T$ and TMN-18 clustered into the '*A. psychrolactophilus* group' with strong support (100% bootstrap value). The ML phylogenetic tree showed a similar topology to that of the NJ tree. The similarity of the concatenated sequences between the two novel strains was 99.9%. The similarities between the HLT2-12-2$^T$ strain and the *A. alpinus* S6-3$^T$, *A. psychrolactophilus* B7$^T$ and *A. psychrochitiniphilus* GP3$^T$ strains were 92.54, 90.73 and 88.67%, respectively. These values are in the range of those of recognized species (98.66–73.40%) that are present in the tree, and thus support the classification of the two strains within the genus *Arthrobacter*, representing a putative novel species.

Strains HLT2-12-2$^T$ and TMN-18, and five reference strains in the '*A. psychrolactophilus* group' were subjected to genome sequencing. The genome size of strain HLT2-12-2$^T$ was about 4.28 Mb. The DNA G + C content of strain HLT2-12-2$^T$ was 61.3 mol%, as determined from the draft genome sequence. Strains HLT2-12-2$^T$ and TMN-18 shared a 99.12% ANI value with each other, and less than 80% ANI value with respect to the closest related species (Table S4), indicating that the two strains indeed are a novel species of the genus *Arthrobacter*.

Strain HLT2-12-2$^T$ contained MK-9($H_2$) as the predominant menaquinone, with a small amount of MK-8($H_2$) and MK-10($H_2$).

**Table 7**
Protologue for *Arthrobacter glacialis* sp. nov.

| | |
|---|---|
| Taxonumber | TA00353 |
| Species name | *Arthrobacter glacialis* |
| Genus name | *Arthrobacter* |
| Species status | sp. nov. |
| Species etymology | gla.ci.a′lis. L. masc. adj. glacialis referring to the glacier environment, the isolation source of the type strain |
| Designation of the type strain | HLT2-12-2 |
| Strain collection numbers | CGMCC 1.10025, NBRC 113092 |
| 16S rRNA gene accession number | JX949500 |
| Genome accession number | PPXC00000000 |
| Genome status | Draft |
| Genome size | 4.28 Mb |
| GC mol % | 61.3–61.5 |
| Country of origin | China |
| Region of origin | Hailuogou glacier in Sichuan province |
| Source of isolation | Glacier ice |
| Sampling date | 01 January 2010 |
| Number of strains in study | 2 |
| Source of isolation of non-type strains | Glacier ice |
| Grouth medium, incubation conditions | Medium [TSA (BD Difco)], temperature [25 °C], pH [7.0] |
| Gram stain | POSITIVE |
| Cell shape | Rod |
| Cell size (length or diameter) | 0.7–1.0 μm in width and 1.4–1.9 μm in length |
| Motility | Nonmotile |
| Colony morphology | Creamy yellow, convex, round (0.5–1.0 mm in diameter) |
| Temperature range | 4–25 °C |
| Lowest temperature for growth | 4 °C |
| Highest temperature for growth | 25 °C |
| Temperature optimum | 20–25 °C |
| Lowest pH for growth | 7 |
| Highest pH for growth | 8 |
| pH optimum | 7 |
| pH category | Neutrophile |
| Lowest NaCl concentration for growth | 0 |
| Highest NaCl concentration for growth | 4 |
| Salinity optimum | 0.5–2 |
| Relationship to $O_2$ | aerobe |
| Positive tests with BIOLOG | Dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-taffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, inosine, D-sorbitol, D-mannitol, glycerol, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-gluconic acid, D-glucuronic acid, glucuronamide, *p*-hydroxy-phenylacetic acid, methyl pyruvate, L-lactic acid, citric acid, L-malic acid, bromo-succinic acid, γ-amino butryric acid, α-hydroxy butyric acid, α-keto-butyric acid, acetoacetic acid, propionic acid, acetic acid, 1% sodium lactate, D-serine, guanidine HCl, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate and sodium bromate. |
| Negative tests with BIOLOG | 3-Methyl glucose, D-fucose, L-rhamnose, D-arabitol, myo-inositol, D-glucose- 6-$PO_4$, D-fructose-6-$PO_4$, D-aspartic acid, gelatin, pectin, D-galacturonic acid, L-galactonic acid lactone, mucic acid, quinic acid, D-saccharic acid, D-lactic acid methyl ester, α-keto-glutaric acid, D-malic acid, Tween 40, β-hydroxy-D,L-butyric acid, formic acid, fusidic acid, troleandomycin, rifamycin SV, minocycline, lincomycin, niaproof 4, vancomycin, tetrazolium violet and tetrazolium blue. |
| Positive tests with API | ONPG, CIT, URE, VP, GEL, NO3, ESC, PNPG |
| Negative tests with API | ADH, LDC, ODC, H2S, TDA, IND, TRP, GLU |
| Commercial kits used | BIOLOG GEN III, API 20E, API 20NE |
| Energy metabolism | Chemoorganotroph |
| Oxidase | Negative |
| Catalase | Positive |
| Negative tests | Dose not hydrolyze starch, casein and Tween 80. |
| Quinone type | MK-9($H_2$) |
| Major fatty acids | anteiso-$C_{15:0}$ (71.9%), anteiso-$C_{17:0}$ (12.6%), iso-$C_{16:0}$ (6.6%), $C_{16:0}$ (3.6%) and iso-$C_{15:0}$ (2.6%) |
| Peptidoglycan type | A3α (L-Lys-L-Thr-L-Ala3; A11.28) |
| Phospolipid pattern or diagnostiv phospolipid | Phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and one unidentified lipid |
| Glycolipids | Dimannosylglyceride |
| Biosafety level | 1 |

Its polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, dimannosylglyceride and one unidentified lipid (Fig. S5). The menaquinone and polar lipids characteristics of strain HLT2-12-2$^T$ are in line with those of the '*A. psychrolactophilus* group' [8]. The total hydrolysate of peptidoglycan contained threonine, glutamic, alanine and lysine. The partial hydrolysate contained Ala–Lys, Ala–Glu, Lys–Thr, Ala–Lys–Thr and $Ala_{3-4}$. Accordingly, we inferred that the peptidoglycan type of strain HLT2-12-2$^T$ was A3α (L-Lys-L-Thr-L-$Ala_3$; A11.28), which was also present in strains *A. alpinus* S6-3$^T$ and *A. psychrolactophilus* B7$^T$ [8,47]. The whole-cell sugars included rhamnose, galactose, ribose and glucose. The fatty acid profile of strains HLT2-12-2$^T$ and TMN-18 were similar to that of the reference strains (Table S5). The cellular fatty acid content of strain HLT2-12-2$^T$ was as follows (>2%): anteiso-$C_{15:0}$ (71.9%), anteiso-$C_{17:0}$ (12.6%), iso-$C_{16:0}$ (6.6%), $C_{16:0}$ (3.6%) and iso-$C_{15:0}$ (2.6%).

Cells of strain HLT2-12-2$^T$ were irregular rods showing a rod–coccus cycle, aerobic, Gram-stain-positive, non-motile, 0.7–1.0 × 1.4–1.9 μm after 3 days on TSA agar at 25 °C. The differential phenotypic characteristics between strains HLT2-12-2$^T$,

TMN-18 and the reference strains are shown in Table 6. The two novel strains can be easily differentiated from the type strains of their closest related species by the 16S rRNA and housekeeping gene sequences, ANI values and phenotypic characteristics. Therefore, they should be classified as a novel species of the genus *Arthrobacter*, for which the name *A. glacialis* sp. nov. is proposed. Strain HLT2-12-2$^T$ (= CGMCC 1.10025$^T$ = NBRC 113092$^T$) is the type strain. The full description of the new taxon is shown in Table 7, as obtained from the Digital Protologue database website (http://imedea.uibcsic.es/dprotologue/) in which the new species was registered under reference TA00353.

## Acknowledgements

This study was supported by grants nos. 31670003 and 31600007 from the National Natural Science Foundation of China (NSFC) and grants no. 2015FY110100 from National Science and technology foundation project.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.10.005.

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