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Phylogenetic diversity of culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China

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Abstract

Microbes have been discovered in permafrost sediments for nearly a century. However, microbiological analyses of alpine permafrost are very scarce. This study is a first attempt to describe the phylogenetic diversity of a culturable bacterial community isolated from alpine permafrost in the Tianshan Mountains in northwestern China. Aerobic 2.5–6.0 $\times 10^5$ CFU/gdw (CFU per 1 gram of dry weight) on modified PYGV medium were recovered from alpine permafrost samples at 4 °C; among these, 91 bacterial isolates with different morphotypes were characterized by phenotypic properties, such as morphology, colony pigmentation, Gram staining, endospore formation and temperature range of growth. The isolates were further categorized based on amplified rDNA restriction analysis (ARDRA), and 51 representative isolates possessing distinct ARDRA patterns selected for subsequent 16S rDNA sequencing and phylogenetic analysis. The phylogenetic trees placed the 51 isolates in four major groups: the high-G + C Gram-positives, the low-G + C Gram-positives, *Proteobacteria* and the *Cytophaga–Flavobacterium–Bacteroides* (CFB) phylum. The most abundant and diverse isolates were members of Gram-positive bacteria, particularly the *Arthrobacter* as a dominant group in alpine permafrost culturable populations. Results of the Jukes–Cantor evolutionary distance matrix suggested that the vast majority of the isolates were different strains of known species, and three may represent new species within the genus *Chryseobacterium* of the CFB phylum. From this study, it is proposed that alpine permafrost sediments in the Tianshan Mountains provide a specific ecological niche for prolonging survival of diverse microbial lineages.

Keywords: Alpine permafrost; Bacterial diversity; 16S rDNA

1. Introduction

Mid-latitude alpine permafrost in the source area of the Urumqi River of the Tianshan Mountains is a unique extreme habitat where the lower limits of alpine permafrost are 3100–3400 m [42]. Snowfall in winter and early spring resists ground cooling, while that in summer preserves frozen ground [34]. Thus, alpine permafrost in the Tianshan Mountains is a stable cold environment with an average temperature of −5 °C. Moreover, alpine permafrost in the Tianshan Mountains, in contrast to ancient Siberian and Polar permafrost deposits, has been characterized by its morphology which underwent numerous changes during the Pleistocene and the Holocene [20]. At the end of the Pleistocene, the lower limits of permafrost in the Tianshan Mountains were 1200–1500 m lower than they are today [19]. During the Holocene, these altitudinal boundaries moved up and down alternatively. Climatic changes in the source area of the Urumqi River, Xinjiang have been comparatively mild since the last glaciation, and syngenetic permafrost has formed as a result. At present, the upper Urumqi River area is still periglacial.

Since Omelyansky first reported the presence of viable microbes in permafrost in 1911, the viable microorganisms inhabiting permafrost represent a significant part of the biosphere [31]. In initial studies, James and Sutherland (1942) found the existence of viable aerobic and anaerobic bacteria in permafrost in northern Canada [24]. In 1961, Becker and Volkmann successfully isolated four genera of bacteria

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from Arctic permafrost samples with 6–18 m depth near Fairbanks, Alaska [5]. Boyd and Boyd subsequently performed the first quantitative investigation of permafrost microorganisms in the western arctic soils and found thermophilic microbes in the permafrost samples [8]. Viable bacteria from Antarctic permafrost were first found by Cameron and Morelli (1974) in association with the Antarctic Dry Valley Drilling Project [9]. Although microbes have been resuscitated from permafrost deposits sampled around the globe, descriptions of alpine permafrost microbial communities are limited and their existence in the Tianshan Mountains has not been previously explored.

Most studies have focused on microorganisms trapped in permafrost, including the culturing and counting of microbial cells from Siberian permafrost, isolating large numbers of various physiological types of permafrost microorganisms and characterizing the phylogenetic diversity of viable bacterial communities from permafrost samples by 16S rDNA sequence analysis. In the 1990's, Russian researchers found that the total number of microorganisms in Siberian permafrost was up to 10^8 cells/g and the number of culturable microbes was in the range of 10^2–10^8 cells/g [16,17,26,44]. The microbial community in permafrost, composed mainly of aerobic psychrotrophic prokaryotes, has been described as "a community of survivors" [14,18,21]. Scientists have known for nearly a century that microorganisms can survive in permafrost environments, and numerous aerobic chemoheterotrophs, strict anaerobic sulfate-reducing, nitrifying and denitrifying bacteria have been isolated from permafrost sediments [40,43]. In the present study, basic investigations into culturable bacteria from alpine permafrost in the Tianshan Mountains, including isolation, phenotypic traits and phylogenetic diversity analysis, have been carried out.

With the advancement of molecular biological means of identifying extremophiles, molecular biological and cultivation-independent approaches for describing microbial diversity have opened up new perspectives for microbial ecology and have been widely used in the searches for microbial communities in extremely cold environments in addition to permafrost [7,10,30]. Although these approaches avoid the limitations of traditional culture techniques, further physiological research on extremophiles and relevant biotechnological developments will require isolation and cultivation of microbes in various extreme cold environments. Recently, increasing attention has been paid to the study of cold-active enzymes [1,2,6,11,13, 15,23,32]; alpine permafrost microorganisms, as potentially useful sources of cold-active enzymes, will play an important role in such studies. Thus, bacterial diversity associated with alpine permafrost from the Tianshan Mountains has been investigated using the molecular technique of targeting 16S rDNA sequences based on culture procedures. These valuable species isolated from alpine permafrost sediments are not only an invaluable part of microbial biodiversity, but will provide us with good candidates for elucidating microbial cold adaptation mechanisms and for exploring their possible biotechnological applications.

2. Materials and methods

2.1. Collection and chemical characterization of alpine permafrost samples

An alpine permafrost core with 3 m depth was collected at the mouth of the ice-free cirque in the Tianshan Mountains (43°07′10.2″ N, 86°49′28.2″ E, 3833 m) in September 2004 (Fig. 1). At this site, alpine permafrost sediments were developed from moraine deposits which had accumulated during the late stage of the Last Glaciation [41], and the depth of alpine permafrost table is shallower than that of other areas of the Tianshan Mountains. The seasonal thaw starts in June and reaches maximal depth in September. Permafrost samples were obtained using the previously described methods of sampling without drilling fluid to avoid contamination with non-indigenous microbes [26]. It was important that the extracted core always remained frozen; the surface material of the core was trimmed away with sterile knives and the core was split into 8-cm-long sections, which were placed in presterilized aluminum tins, sealed and kept frozen during storage and transport. In the laboratory the samples were stored at −20 °C. Immediately after sampling, the chemical composition and the pH of this alpine permafrost core were measured by Dionex DX-600 (USA) and Dionex ICS-2500 (USA) ion chromatography (Table 1).

2.2. Isolation and culture

A recovery protocol was used to initially isolate permafrost microorganisms: direct plating of diluted cell suspensions was conducted on the recovery medium. The modified PYGV agar medium (0.2% yeast extract, 0.4% peptone, 0.4% glucose and other components similar to DSMZ Medium 621: PYGV AGAR) was used as the recovery medium according to many preexperiments, and the chemical characteristics of alpine permafrost samples [37]. Sediment samples were diluted by aseptically weighing 1 g of wet sediment into a 250 ml flask containing 99 ml sterile physiological salt solution with glass beads, and shaking for 30 min at 4 °C. Serial dilutions were plated on prechilled modified PYGV medium and incubated aerobically at 4 °C for two weeks. Distinct colony types on the spread plates of each 8-cm-long section were purified by streaking and restreaking on PYG (0.2% yeast extract, 0.4% peptone, and 0.4% glucose) agar medium. Then the purified isolates were subcultured on PYG plates at 4 °C, and the cells were stored at −70 °C in a 15% glycerol solution.

2.3. Phenotypic characterization of the isolates

A total of 91 aerobic colonies from each morphotype were selected from the modified PYGV recovery plates and assessed on the basis of phenotypic traits. Gram staining, endospore formation, colony morphology and pigmentation were performed as described previously [38]. The temperature growth range of the isolates was conducted on PYG agar medium at 0, 4, 12, 20, 24, 30, and 37 °C. The cell morphological characteristics of the

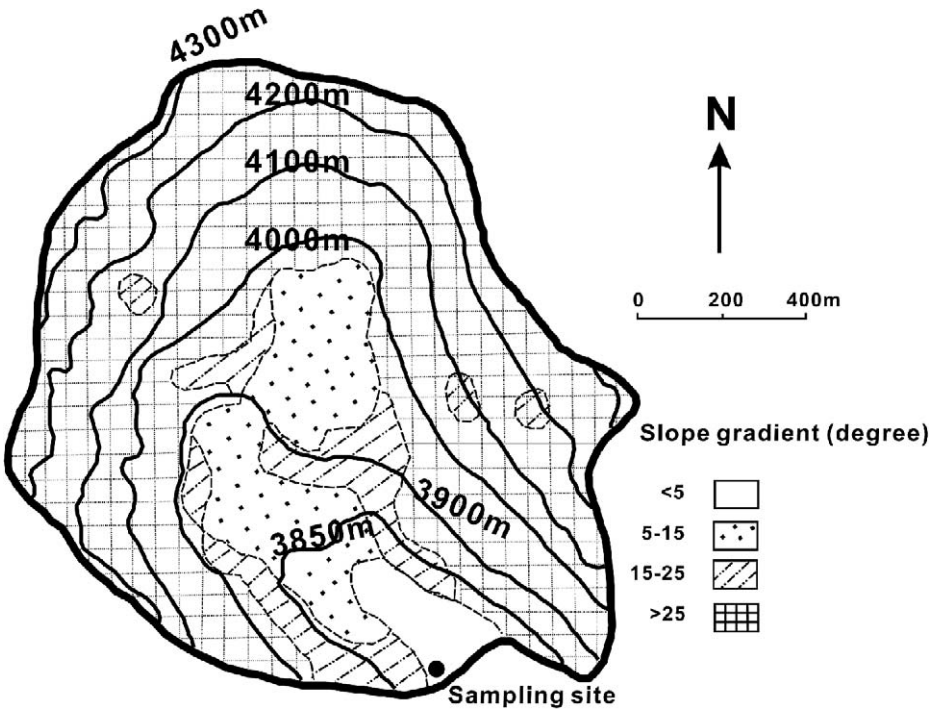

Fig. 1. Sampling site on map of inclination of ice-free cirque drawn by Z. Bai [3].

Table 1
Chemical composition and pH of alpine permafrost in the Tianshan Mountains

Chemical composition	Concn. (mM/kg)
Cations	
Sodium (Na^+)	1.639
Ammonium (NH_4^+)	0.370
Potassium (K^+)	0.629
Magnesium (Mg^{2+})	0.541
Calcium (Ca^{2+})	3.446
Anions	
Fluoride (F)	0.054
Chloride (Cl^-)	2.860
Nitrate (NO_3^-)	1.108
Sulfate (SO_4^{2-})	3.442
Acetate (CH_3COO^-)	0.082
pH	6.8

isolates were examined by transmission electron microscopy. Cells grown at 12 °C were prepared as in [27]. Samples were negatively stained with 1% phosphotungstic acid (pH 7.0) and observed under a JEM-1230 transmission electron microscope (JEOL, Tokyo).

2.4. PCR amplification and sequencing of 16S rDNA

The colonies grown on PYG agar at 12 °C were suspended in 100 µl sterilized deionized water, and then 2 µl of the suspension was used to amplify the 16S rDNA by bacterial universal primers 8F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′), corresponding to positions 8–27 and 1492–1511, respectively, in the 16S rDNA sequence of *Escherichia coli*. The thermal PCR profile was as follows: initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 40 s, primer annealing at 54 °C for 40 s, elongation at 72 °C for 1.5 min, and the final elongation step was 7 min at 72 °C. The 16S rDNA products were analyzed by electrophoresis on 1% agarose gels. ARDRA analysis was performed using three four-base pair-cleaving restriction endonucleases (*Msp*I, *Hae*III, *Alu*I) at 37 °C for 12 h, and the resulting electrophoretic morphology in 2% agarose were used to group the isolates. The 16S rDNA sequences of all major ARDRA patterns were further purified and sequenced.

2.5. Phylogenetic analysis of the isolates

The partial 16S rDNA sequences of the isolates corresponding to positions 110–1431 in the *E. coli* numbering system were compared with 16S rDNA sequences available by the BLAST search in the National Center for Biotechnology Information (NCBI) database (http://www.nvbi.nlm.nih.gov) [22]. Multiple sequence alignments were performed using Clustal W version 1.8 [39]. The method of Jukes and Cantor (1969) was used to calculate evolutionary distances; phylogenetic dendrograms were constructed by the neighbor-joining method [35] and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 3.1 (Molecular Evolutionary Genetics Analysis).

2.6. Nucleotide sequence accession numbers

The isolates from alpine permafrost samples in the Tianshan Mountains were designated as TSBY1, TSBY2 and so on. The partial 16S rDNA sequences of 51 representatives were deposited in GenBank and their accession numbers were given in parentheses after the isolate number: TSBY 7 (DQ172982),

TSBY 9 (DQ172984), TSBY 11 (DQ145757), TSBY 12 (DQ172986), TSBY 14 (DQ145940), TSBY 18 (DQ166179), TSBY 20 (DQ172990), TSBY 21 (DQ172991), TSBY 22 (DQ172992), TSBY 23 (DQ172993), TSBY 24 (DQ150099), TSBY 25 (DQ172994), TSBY 26 (DQ172995), TSBY 28 (DQ172997), TSBY 35 (DQ173000), TSBY 37 (DQ173002), TSBY 38 (DQ151830), TSBY 39 (DQ173003), TSBY 43 (DQ173006), TSBY 44 (DQ151832), TSBY 45 (DQ173007), TSBY 46 (DQ173008), TSBY 49 (DQ151834), TSBY 50 (DQ173010), TSBY 51 (DQ173011), TSBY 55 (DQ151836), TSBY 56 (DQ151837), TSBY 57 (DQ173014), TSBY 58 (DQ173016), TSBY 59 (DQ173017), TSBY 61 (DQ166168), TSBY 62 (DQ173018), TSBY 63 (DQ173019), TSBY 64 (DQ173020), TSBY 65 (DQ173021), TSBY 66 (DQ173022), TSBY 67 (DQ166169), TSBY 69 (DQ173023), TSBY 70 (DQ166171), TSBY 74 (DQ173026), TSBY 79 (DQ173030), TSBY 82 (DQ173032), TSBY 84 (DQ166174), TSBY 85 (DQ166175), TSBY 86 (DQ166176), TSBY 88 (DQ173034), TSBY 89 (DQ173035), TSBY 90 (DQ173036), TSBY 91 (DQ166177), TSBY 92 (DQ173037), TSBY 93 (DQ166178).

3. Results

3.1. Phenotypic characterization of the isolates

After aerobic incubation for two weeks at 4 °C, visible 2.5–6.0 × 10^5 CFU/gdw (CFU per 1 gram of dry weight) on the modified PYGV agar plates were recovered from alpine permafrost samples of each 8-cm-long section, among which 91 colonies with distinct morphotypes were characterized by phenotypic properties such as Gram staining, morphology, colony pigmentation, sporulation, and temperature range of growth (Fig. 2 and Table 2).

Among them, 68 isolates (74.7%) were rod-shaped and 23 (25.3%) were cocci. This result is consistent with the previous report that bacilli are dominant in Siberian permafrost deposits [36]. Among the 91 isolates we studied, 55 (60.4%) were Gram-positive and 36 (39.5%) Gram-negative. 42.9% of the isolates were Gram-positive rod-shaped and 39.6% were observed to form pigmented colonies (peachblow, yellow, pink, beige and orange). Only TSBY79, related to the genus *Trichococcus,* was an endospore former.

According to the definition by Morita (1975), most of the isolates (89%) from alpine permafrost deposits were psychrotrophic and could not grow at temperatures above 30 °C, but often grew at temperatures about 4 °C or below. Only two isolates (TSBY62 and TSBY91) were psychrophilic and could grow at 0 °C, with optimal growth occurring at about 12 °C and the upper limit being 20 °C. A few isolates had an optimum temperature for growth at about 24 °C and grew over a wide temperature range of 4–37 °C and even 0–37 °C.

3.2. Phylogenetic analysis of 16S rDNA sequences of isolates

From 91 isolates having visibly different colony morphologies, 51 representatives with diverse ARDRA patterns (data not shown) were selected for subsequent 16S rDNA sequencing and phylogenetic analysis (Table 2). Phylogenetic analysis of 51 16S rDNA sequences indicated that the isolates belonged to four major groups: the high-G + C Gram-positives, the low-G + C Gram-positives, *Proteobacteria*, and the CFB phylum.

The most abundant and diverse isolates belonged to the high-G + C Gram-positives group, the *Arthrobacter* in particular, as a dominant subgroup in the high-G + C Gram-positives (Fig. 3). 16S rDNA sequence phylogenetic analysis of eighteen isolates related (>97.9%) to the genus *Arthrobacter* confirmed that these isolates presented a more dispersed relationship with several species, namely, *Arthrobacter psychrophenolicus*, *Arthrobacter sulfurous*, *Arthrobacter kerguelensis*, *Arthrobacter gangotriensis*, *Arthrobacter polychromogenes*, *Arthrobacter oxydans*, *Arthrobacter psychrolactophilus*, *Arthrobacter citreus*. Six other isolates were affiliated with different genera in the linage of the Microbacteriaceae, namely, *Clavibacter*, *Frigoribacterium*, *Okibacterium*, *Leifsonia* and *Rhodococcus*, with more than 97.2% sequence similarity. Only TSBY82 was most closely related to the lineage of the Nocardiaceae of the suborder Corynebacterineae, with 100% sequence similarity to *Rhodococcus erythropolis* (AY785731). Three isolates were members of different genera of the low-G + C Gram-positives (Fig. 3). TSBY85 showed the highest sequence similarity (99.6%) with *Exiguobacterium* sp. B01 (AB219055). TSBY25 was closely related to *Planococcus psychrotoleratus* (AF324659), with 99.6% sequence similarity. TSBY79 had 99.8% sequence similarity to *Trichococcus palustris* (X87150).

The next most abundant and relatively diverse of the organisms belonging to *Proteobacteria* fell into three major lineages: alpha, beta and gamma subdivisions (Fig. 4). All five isolates belonging to the alpha subdivision of *Proteobacteria* were affiliated with the genus *Sphingomonas*. TSBY64 and TSBY38 had 99.4 and 99.8% sequence similarity, respectively, to *Sphingomonas* sp. Enf2 (DQ339610) and *Sphingomonas aurantiaca* (AJ429237). The remaining three isolates (TSBY49, TSBY61 and TSBY92) were clustered together, among which TSBY61 and TSBY92 with 99.0% sequence similarity to each other showed 100 and 99.0% sequence similarity to *Sphingomonas* sp. XT-11 (DQ115797), respectively. TSBY49 had *Sphingomonas* sp. J05 (AJ864842) as the nearest neighbor, with 99.9% sequence similarity.

Three isolates were affiliated with the beta-subdivision of *Proteobacteria*. TSBY86 was most closely related to *Janthinobacterium lividum* (AF174648) (99.8% sequence similarity). TSBY55 was most similar (98.1%) to glacial ice bacterium CanDirty89 (AF479326) reported by Christner. Although detailed phylogenetic affiliation of this strain was not discussed in their report, TSBY55 can be considered to belong to *Janthinobacterium agaricidamnosum* (Y08845), with 98% sequence similarity. The remaining one, TSBY44, showed 99.8% sequence similarity to *Massilia* sp. CAI-19 (DQ257420).

Seven isolates related to the gamma subdivision of *Proteobacteria* formed two subclusters supported by 100% bootstrap confidence values. Four isolates in one subcluster were affiliated with two species of the genus *Psychrobacter*. Among them, TSBY91 and TSBY70 had more than 99.9% sequence similarity to *Psychrobacter maritimus* (AJ609272) and *Psy-*

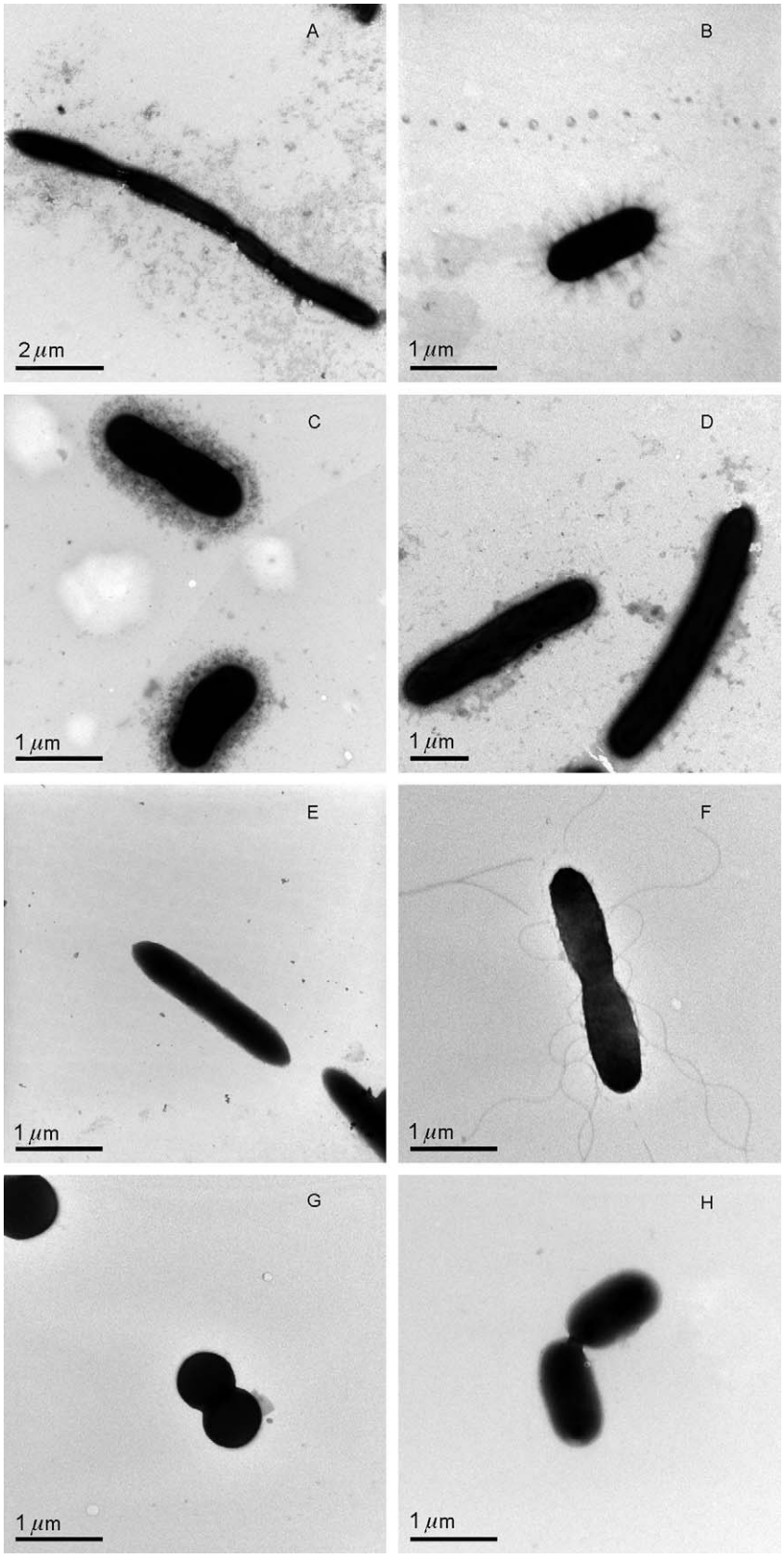

Fig. 2. TEM images of culturable aerobic bacteria isolated from alpine permafrost samples in the Tianshan Mountains: (A) Gram-negative, non-flagellated, non-spore-forming and long thin rod-shaped TSBY11. (B) Short rod-shaped TSBY30. (C) Gram-negative short rod-shaped TSBY49. (D) Long rod-shaped TSBY2. (E) Rod-shaped TSBY14 having pointed ends. (F) Gram-negative rod-shaped TSBY86 with lateral flagella. (G) Actively dividing Gram-positive coccoid-shaped TSBY52. (H) Short thick rod-shaped TSBY13.

Table 2
Characteristics of selected isolates from alpine permafrost in the Tianshan Mountains

Isolate No.	Cell and colony morphology	Growth temp. (°C)	Closest relative species in the 16S rDNA sequence database (GenBank accession No.)	Similarity (%)
Gram-positive group				
TSBY 7	Cream, small; rod-shaped	4-24[a]-37	*Arthrobacter* sp. An16 (AJ551154)	98.3
TSBY 9	Translucent white, large; rod-shaped	4-20-30	*Microbacterium* sp. VA8728_00 (AF306834)	97.5
TSBY 18	Strong yellow, small; rod-shaped	0-20-30	*Frigoribacterium faeni* (Y18807)	99.7
TSBY 20	Cream, small; rod-coccus cycle	0-20-30	*Arthrobacter* sp. SB (AY327445)	99.9
TSBY 21	Smooth and pink-cream; rod-shaped	4-20-30	*Arthrobacter* sp. 45-3 (AY444853)	99.9
TSBY 23	Yellowish, small; rod-coccus cycle	4-20-30	*Arthrobacter* sp.An10 (AJ551149)	99.0
TSBY 24	Cream, small; long, thin and rod-shaped	0-20-30	*Arthrobacter* sp. ON14 (AJ810894)	99.8
TSBY 25	Orange, small; coccoid-shaped	4-24-37	*Planococcus psychrotoleratus* (AF324659)	99.6
TSBY 26	Orange-red, small; rod-shaped	0-20-30	*Frigoribacterium* sp. PIC-C17 (DQ227784)	99.9
TSBY 28	White, small; rod-coccus cycle	4-20-30	*Arthrobacter psychrolactophilus* (AF134180)	98.5
TSBY 35	Smooth and pink-cream; rod-shaped	4-20-30	*Arthrobacter* sp. CK22 (AJ920001)	100
TSBY 43	Cream, small; rod-shaped	4-20-30	*Arthrobacter* sp. 45-3 (AY444853)	99.9
TSBY 45	Translucent white, large; rod-shaped	4-20-30	*Arthrobacter* sp. 45-3 (AY444853)	99.0
TSBY 46	Lemon yellow; rod-shaped	4-20-30	*Arthrobacter citreus* (X80737)	99.7
TSBY 50	Yellow, medium; rod-coccus growth cycle	0-20-30	*Arthrobacter* sp. Tibet-ITa1 (DQ108398)	99.8
TSBY 58	Cream, large; rod-coccus cycle	4-20-30	*Arthrobacter* sp. ON14 (AJ810894)	99.2
TSBY 59	Yellowish, medium; rod-shaped	4-20-30	*Arthrobacter gangotriensis* (AJ606061)	99.5
TSBY 63	Smooth and pink-cream; rod-shaped	4-20-30	*Arthrobacter psychrolactophilus* (AF134180)	99.1
TSBY 65	Cream, small; rod-shaped	4-20-30	*Arthrobacter* sp. ON14 (AJ810894)	99.3
TSBY 69	Pale white, large; rod-coccus cycle	4-20-30	*Arthrobacter psychrolactophilus* (AF134180)	99.5
TSBY 74	Light orange-red, small; rod-shaped	4-20-30	*Clavibacter* sp. Enf56 (DQ339617)	100
TSBY 79	Snowy white, small; rod-shaped	4-24-37	*Trichococcus pasteurii* (X87150)	99.8
TSBY 82	Cream, medium; rod-shaped	4-20-30	*Rhodococcus erythropolis* (AY785731)	100
TSBY 84	Translucent white, small; rod-shaped	4-20-30	*Microbacterium* sp. VA8728_00 (AF306834)	97.2
TSBY 85	Orange-red, small; rod-shaped	4-20-30	*Exiguobacterium* sp. B01 (AB219055)	99.6
TSBY 88	Smooth and pink-cream; rod-shaped	4-20-30	*Arthrobacter* sp. Amico7 (AY512633)	100
TSBY 89	Snowy white, small; rod-shaped	4-20-30	*Rhodoglobus* sp. GICR18 (AY439269)	97.5
TSBY 90	Cream, medium; rod-shaped	4-20-30	*Arthrobacter* sp. ON14 (AJ810894)	99.3
Proteobacteria group				
TSBY 37	White, large; coccoid-shaped	0-20-30	*Psychrobacter fozii* (AY771717)	99.6
TSBY 38	Orange-red, small; rod-shaped	4-20-30	*Sphingomonas aurantiaca* (AJ429237)	99.8
TSBY 44	Translucent yellow, small; rod-shaped	4-20-30	*Massilia* sp. CAI-19 (DQ257420)	99.8
TSBY 49	Orange, small; rod-shaped	4-20-30	*Sphingomonas* sp. J05 (AJ864842)	99.9
TSBY 51	Smooth and pink-cream; rod-shaped	4-20-30	*Pseudomonas* sp. PD 26 (DQ377767)	99.0
TSBY 55	Pale yellow, small; rod-shaped	0-20-30	*Janthinobacterium agaricidamnosum* (Y08845)	98.0
TSBY 61	Orange-red, small; coccoid-shaped	4-20-30	*Sphingomonas* sp. XT-11 (DQ115797)	100
TSBY 62	White, large; coccoid-shaped	0-12-20	*Psychrobacter fozii* (AY771717)	99.8
TSBY 64	Yellow, small; rod-shaped	4-20-30	*Sphingomonas* sp. Enf2 (DQ339610)	99.4
TSBY 66	Smooth and pink-cream; rod-shaped	4-20-30	*Pseudomonas* sp. PD 26 (DQ377767)	99.4
TSBY 70	White, large; coccoid-shaped	0-20-30	*Psychrobacter* sp. 9B_7 (AY689064)	100
TSBY 86	Smooth and violet pigmented; rod-shaped	0-24-37	*Janthinobacterium* sp. J31 (AJ864846)	99.8
TSBY 91	Smooth and pink-cream; coccoid-shaped	0-12-20	*Psychrobacter maritimus* (AJ609272)	99.9
TSBY 92	Orange-red, small; rod-shaped	4-20-30	*Sphingomonas* sp. XT-11 (DQ115797)	99.0
TSBY 93	Smooth and pink-cream; long rod-shaped	4-20-30	*Pseudomonas frederiksbergensis* (AY785733)	100
CFB group				
TSBY 11	Pale yellow, smooth; long rod-shaped	0-20-30	*Flavobacterium xinjiangense* (AF433172)	99.3
TSBY 12	Smooth and pink-cream, slimy; rod-shaped	4-20-30	*Pedobacter* sp. TB4-9-II (AY599663)	99.8
TSBY 14	Pale white, large, slimy; rod-shaped	4-20-30	*Pedobacter* sp. TB4-9-II (AY599663)	99.8
TSBY 22	Smooth and pink-cream, slimy; rod-shaped	4-20-30	*Pedobacter* sp. TB4-9-II (AY599663)	99.5
TSBY 39	Orange-yellow, large; rod-shaped	0-20-30	*Chryseobacterium soldanellicola* (AY883415)	95.1
TSBY 56	Peachblow, small; rod-shaped	4-20-30	*Pedobacter aurantiacus* (DQ235228)	97.0
TSBY 57	Yellow, slimy; rod-shaped	0-20-30	*Chryseobacterium soldanellicola* (AY883415)	95.1
TSBY 67	Orange, small; rod-shaped	4-24-37	*Chryseobacterium soldanellicola* (AY883415)	96.7

[a] The optimal growth temperature.

chrobacter sp. 9B_7 (AY689064), respectively. The other two isolates (TSBY62 and TSBY37) had 99.4% sequence similarity to each other and showed 99.8 and 99.6% sequence similarity to *Psychrobacter fozii* (AY771717), respectively. In the other subcluster, TSBY51, TSBY66 and TSBY93 were within the genus *Pseudomonas*, among which TSBY66 and TSBY51 had *Pseudomonas* sp. PD 26 (DQ377767) as the nearest neighbor, with 99.4 and 99.0% sequence similarity, respectively. TSBY93 showed 100% sequence similarity to *Pseudomonas frederiksbergensis* (AY785733).

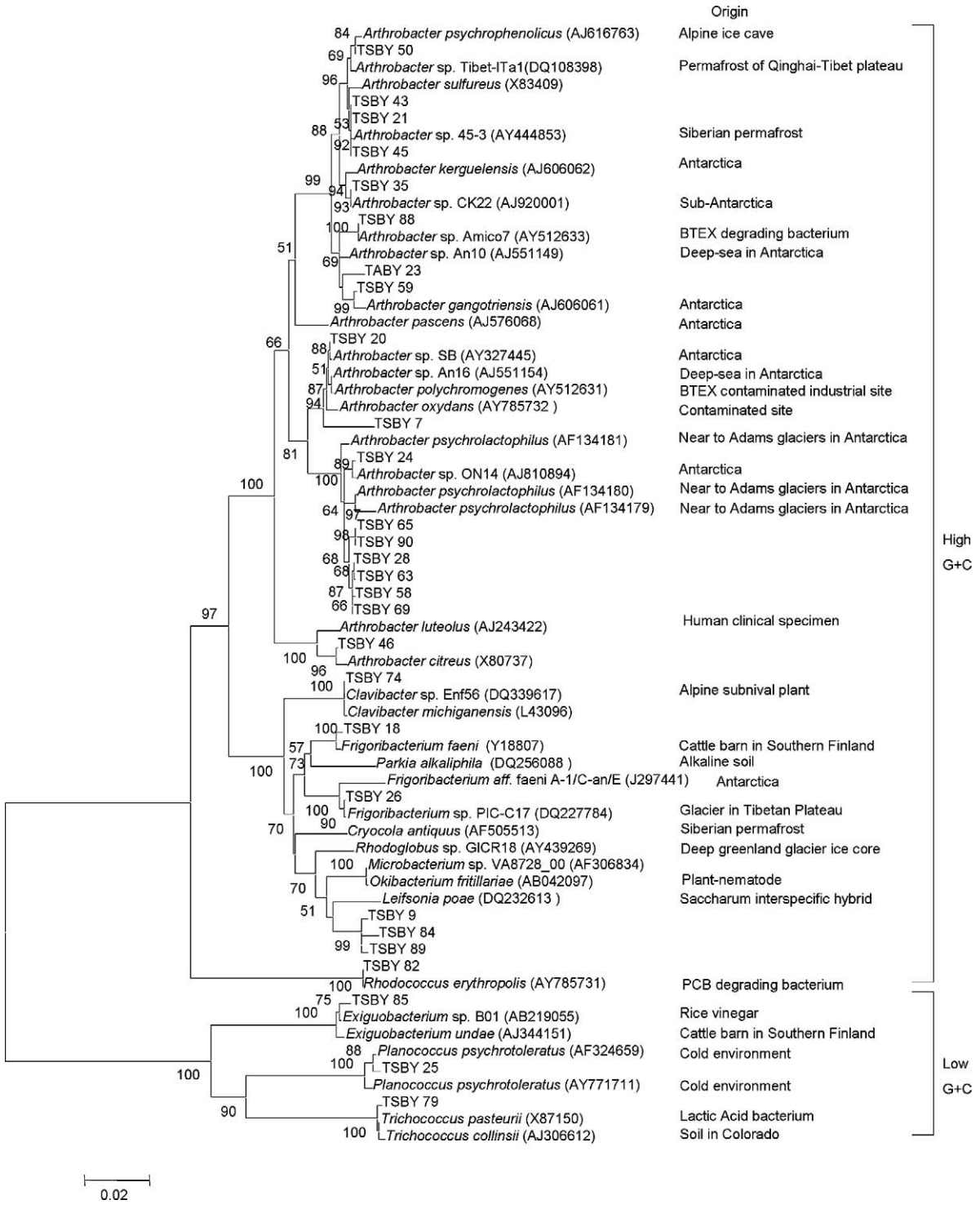

Fig. 3. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of Gram-positive representatives and their closest phylogenetic relatives. Only >50% bootstrap values ($n = 1000$ replications) were indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

Eight isolates belonging to the CFB phylum were less diverse and particularly concentrated in the *Cytophaga–Flavobacteria* complex, including the classes *Flavobacteria* and *Sphingobacteria* (Fig. 5). TSBY67 had only 96.7% sequence similarity to *Chryseobacterium soldanellicola* (AY883415), indicating that TSBY67 was possibly a new taxon within the *Chryseobacterium* group of the CFB phylum. TSBY39 and TSBY57 formed a single cluster in the tree and showed 95.1% sequence similarity to *Chryseobacterium soldanellicola* (AY883415), indicating that they may also represent new species within the genus *Chryseobacterium*. TSBY11 had 99.3% sequence similarity to *Flavobacterium xinjiangense* (AF433172). The remaining four isolates (TSBY12, TSBY14, TSBY22, and TSBY56) were clustered with the *Pedobacter* group of the CFB phylum and all but TSBY56 branched singly within this group with high similarity (98.0–98.8%) to *Pe-*

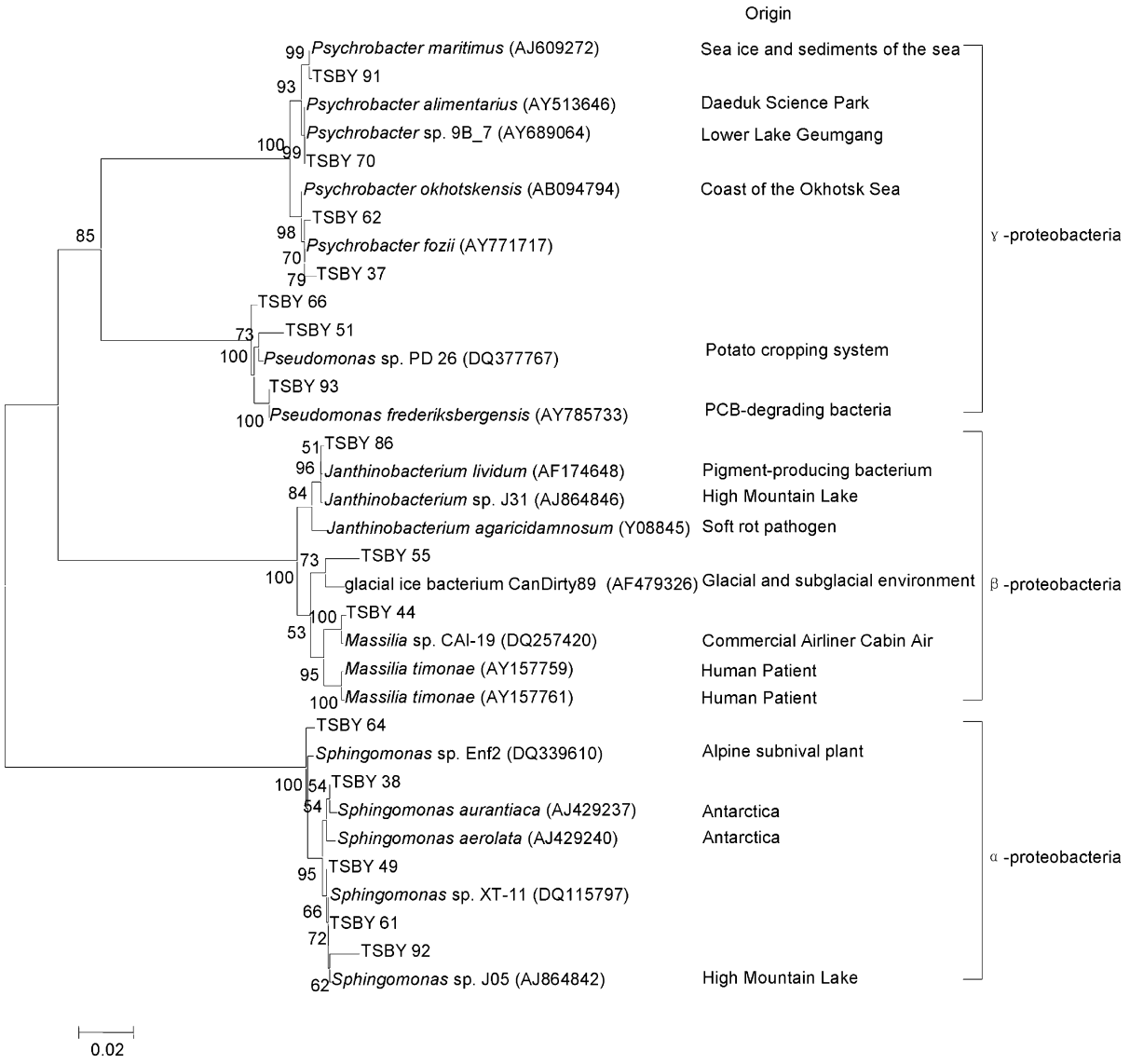

Fig. 4. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of representative isolates affiliated with *Proteobacteria* and their closest phylogenetic relatives. Only >50% bootstrap values ($n = 1000$ replications) were indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

dobacter sp. TB4-9-II (AY599663). TSBY56 was related to *Pedobacter aurantiacus* (DQ235228), with 97.0% sequence similarity.

4. Discussion

Alpine permafrost in the Tianshan Mountains is a unique cold environment with an average temperature of −5 °C, and in which the most abundant ions were Na^+, Ca^{2+}, Cl^-, NO_3^-, and SO_4^{2-}. It is also a new and underexplored niche for microbes. For this environment, microbiological analyses of alpine permafrost are very scarce. Previous reports on the isolation of psychrotrophic or psychrophilic microorganisms had mainly focused on the polar regions and the Siberian permafrost at high latitudes. In this work, we have performed multiple studies on the isolation, phenotypic traits and phylogenetic diversity of culturable bacteria from alpine permafrost samples in the Tianshan Mountains. The results of this study suggest that alpine permafrost at mid-latitude of the Tianshan Mountains is also an exciting microbial resource. The data presented here supplement several recent reports of microbes surviving in alpine environments, and establish the phylogenetic diversity of alpine permafrost microbiota in the Tianshan Mountains.

To better understand the culturable microbial community in alpine permafrost, it is important to identify an isolation protocol that optimizes the recovery of genetically diverse bacterial lineages. In previous studies, permafrost microbes had been isolated and grown at room temperature in nutrient-rich media. In 1997, Shi et al. isolated viable bacteria from Siberian permafrost by PYGV, a medium with low nutrient concentration used to isolate bacteria from Antarctic soils and rocks. According to many preexperiments and the principal characteristics of alpine permafrost samples, we chose modified PYGV medium on which high colony recovery efficiency and abundance were observed after plating and incubation conditions relevant to the

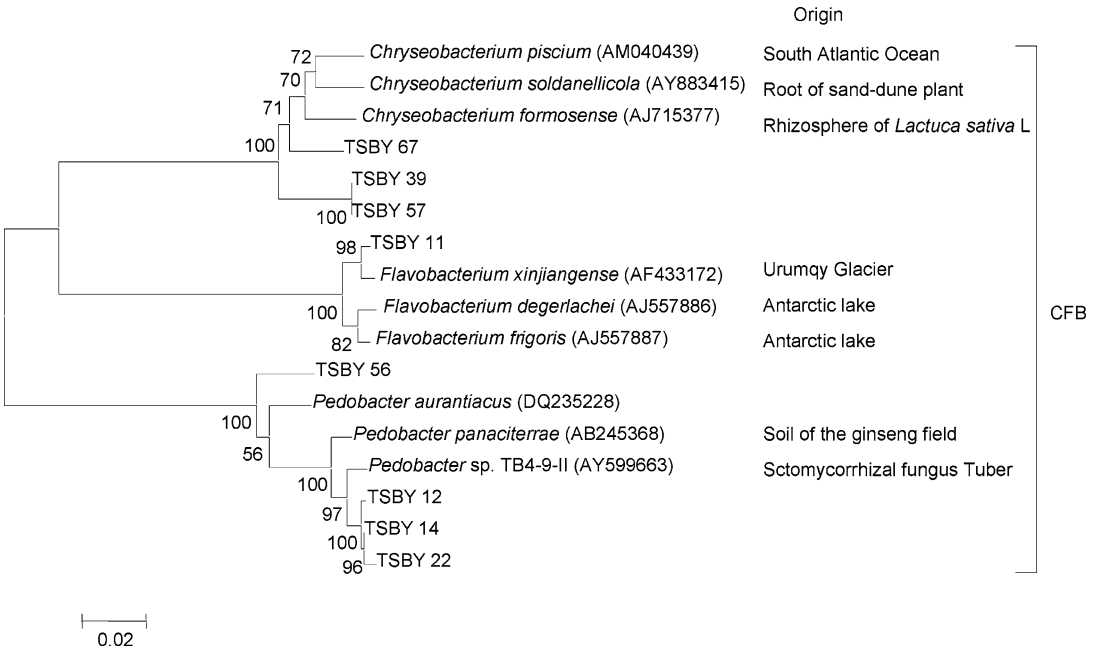

Fig. 5. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of representative isolates related to the CFB phylum and their closest phylogenetic relatives. Only >50% bootstrap values ($n = 1000$ replications) were indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

natural environment so as to maximize the recovery of culturable organisms. Of course, further studies of the survival strategies and conditions will be necessary to explore a multiple microbial ecosystem in alpine permafrost of the Tianshan Mountains.

91 isolates representing different colony morphotypes were categorized by phenotypic characteristics and amplified rDNA restriction analysis (ARDRA), and among them 51 representative isolates were subject to 16S rDNA sequence phylogenetic analysis and found to be affiliated with the genera of *Exiguobacterium*, *Planococcus*, *Trichococcus*, *Chryseobacterium*, *Flavobacterium*, *Pedobacter*, *Sphingomonas*, *Janthinobacterium*, *Massilia*, *Naxibacter*, *Psychrobacter*, *Pseudomonas*, *Arthrobacter*, *Clavibacter*, *Frigoribacterium*, *Okibacterium*, *Leifsonia*, and *Rhodococcus*. Among them, 49 and 29.4% of the isolates belonged to the high G + C Gram-positives and *Proteobacteria*, respectively. Only 15.7% belonged to the CFB phylum and 5.9% to the low-G + C Gram-positives.

All isolates related to the alpha, beta and gamma subdivisions of *Proteobacteria* were a typical and dominant group in cold environments by cultivation methods. However, this study showed that the isolates belonging to the Gram-positives were diverse and predominant in alpine permafrost culturable populations. Within the Gram-positives group, the genus *Arhtrobacter* formed the largest cluster in terms of diversity and high abundance, with 25 representative isolates in 7 phylogenetically distinct subclusters. Although arthrobacters are ubiquitous in many low temperature environments, such as alpine ice caves [29], Antarctica ice shells [28] and Siberian permafrost [36], it is interesting that arthrobacters were dominant members of a culturable microbial community in the alpine permafrost environment of Tianshan. And it is difficult to compare the result observed in this study with those observed in other studies on similar environments because of the scarcity of reports on culturable microbial communities in alpine permafrost.

Most of the isolates were most closely related to bacteria obtained from a variety of cold environments, including ancient Siberian permafrost, high mountain lakes, alpine ice caves and glacial and subglacial environments in the Antarctic [4,11,29,30]. The Jukes–Cantor evolutionary distance matrix was used to calculate the degree of relatedness among alpine permafrost isolates and strains used in constructing the phylogenetic tree. The results suggested that three of the isolates shared <97% 16S rDNA sequence similarity with previously described bacteria and can be considered as new species. All of them (TSBY39, TSBY57 and TSBY67) were related to the CFB phylum that is generally known to degrade complex substrates [12,33], and its isolates are frequently cold-tolerant [25].

Our objective in the present study was to obtain a more complete overview of microbial diversity in alpine permafrost of the Tianshan Mountains, using traditional culture techniques. Our results using modified PYGV medium indicate, first of all, that we can obtain a large number of alpine permafrost microorganisms from a wide variety of phylogenetic groups; indeed, three of the isolates may represent new species within the genus *Chryseobacterium* which are possibly good candidates for further study of their physiology and function in the microbial community. In addition, this investigation has expanded our knowledge of culturable bacterial diversity in cold environments. Although microbes have been found to survive in permafrost sediments for a long period of time, microbial populations in alpine permafrost deposits have not yet been described. In addition, the microbial ecosystem in alpine permafrost plays an important role in the global C cycle, and it is affected by and reflects climate changes. This culture-based

investigation of a microbial community in alpine permafrost deposits has already provided basic information on microbial diversity. Further studies using culture-independent approaches will be necessary to gain a more comprehensive view of biodiversity in alpine permafrost of the Tianshan Mountains.

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