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ORIGINAL PAPER

Diversity and distribution of alkaliphilic psychrotolerant bacteria in the Qinghai–Tibet Plateau permafrost region

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Abstract The Qinghai–Tibet Plateau represents a unique permafrost environment, being a result of high elevation caused by land uplift. And the urgency was that plateau permafrost was degrading rapidly under the current predicted climatic warming scenarios. Hence, the permafrost there was sampled to recover alkaliphilic bacteria populations. The viable bacteria on modified PYGV agar were varied between 10^2 and 10^5 CFU/g of dry soil. Forty-eight strains were gained from 18 samples. Through amplified ribosomal DNA restriction analysis (ARDRA) and phylogenetic analyses, these isolates fell into three categories: high G + C gram positive bacteria (82.3%), low G + C gram positive bacteria (7.2%), and gram negative α-proteobacteria (10.5%). The strains could grow at pH values ranging from 6.5 to 10.5 with optimum pH in the range of 9–9.5. Their growth temperatures were below 37°C and the optima ranging from 10 to 15°C. All strains grew well when NaCl concentration was below 15%. These results indicate that there are populations of nonhalophilic alkaliphilic psychrotolerant bacteria within the permafrost of the Qinhai-Tibet plateau. The abilities of many of the strains to produce extracellular protease, amylase and cellulase suggest that they might be of potential value for biotechnological exploitation.

Keywords 16S rDNA · Alkaliphilic psychrotolerant bacteria · Qinghai–Tibet plateau · Permafrost

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Introduction

Permafrost, defined as ground (including bedrock, soil and sands) that remains below 0°C for more than 2 years (Muller 1943), makes up 26% of the land surface of the Earth (Williams and Smith 1989). Permafrost poses unique challenges to its resident biota because that not only there are only minute amounts of water in the liquid form, little organic compounds and minerals within permafrost but temperatures that have remained below 0°C over geologically significant periods of time and long-term influence of gamma radiation from soil minerals. Hence, the permafrost microbial community has been described as ''a community of survivors'' (Friedmann 1994).

Omelyansky (1911) first reported the presence of viable microbes in permafrost a century ago. Subsequently, microorganisms were recovered from Canada (James and Sutherland 1942), Alaska (Becker and Volkmann 1961; Boyd and Boyd 1964) and south-polar permafrost (Cameron and Morelli 1974). These initial findings aroused further investigations on microbial life in permafrost.

During the latest two decades, most studies focused on the North-Eastern Siberia. With the culture-based method, Shi and his colleagues cultured viable bacteria from the Kolyma lowland permafrost and

characterized them with 16S rDNA sequencing (Shi et al. 1997). Low-temperature recovery strategies were developed and low-nutrient media were used to improve the isolation of bacteria from the ancient permafrost sediment (Vishnivetskay et al. 2000). Besides, viable fungi, actinomycete, methanotrophic and methanogenic archaea were also recovered in permafrost sediments (Gilichinsky and Wagener 1995; Khmelenina et al. 2002). Further, reproduction and metabolism of the isolated bacteria at low temperature were studied (Rivkina et al. 2000; Bakermans et al. 2003). In addition, based on culture-independent methods, the ultrastructure of microbial cells in situ was pictured by electron microscopy methods (Soina et al. 2004), and metabolic activity of permafrost bacteria below the freezing point was measured based on incorporation of ^{14}C-labeled substrates (Rivkina et al. 2000, 2004).

However, while the ''community of survivors'' in high-latitude area was well pictured, we still nearly have no idea of the permafrost microorganisms in a very interesting alpine permafrost region, the Qinghai–Tibet Plateau. The Qinghai–Tibet Plateau is called the 'world's roof' or 'third polar', the highest plateau in the world-average altitude 4,500 m (Cheng 1998). It represents a unique permafrost environment, being a result of high elevation and low latitude caused by land uplift in the order of 3,000 m over the last 200 million years (Wang and French 1995). Permafrost of Qinghai–Tibet Plateau is warm and thin compared with high latitude (polar) permafrost in both North America and Russia, and thus more sensitive to changes in climate and surface conditions (Cheng 1998). As a result, the permafrost degradation was more severe. In the recent 30 years, permafrost areas in the Qinghai–Tibet Plateau were decreased 10,000 km^2 (Li and Cheng 1999). A recent warming trend in ground temperature has been monitored, and, if maintained, permafrost will become relict within the next ca. 150 years (Wang and French 1995). Therefore, investigations into microorganisms from this unique permafrost region were urgent and significant.

During the primary surveys, soils from the Qinghai–Tibet Plateau permafrost region showed the trait of alkaline pH. It therefore seems promising to attempt to isolate the alkaliphiles in this unexplored region for further research and possible relevant biotechnological applications. In this study, primary investigations into the aerobic alkaliphilc bacteria from the Qinghai–Tibet Plateau permafrost region were studied, including their phenotypic traits, phylogenetic diversity and distribution.

Materials and methods

Site description and sampling

The sample sites were located at Beilu-river basin and distributed between 32°24′ and 35°45′N, 91°43′ and 94°20′E (Table 1). The mean annual air temperature (MAAT) here was 5.28°C, and the yearly frozen period was from September to April of the next year. The mean annual rainfall was 290.9 mm, while the evaporation was 1316.9 mm (Niu et al. 2005). It was a permafrost area and 30–50% surface area covered by alpine meadow that was composed of the plants such as *Stipa purpurea*, *Carex moorcroftii*, *C. invanovae*, *Littledalea racemosa*, *Kobresia pygmaea*, *K. robusta*, *Saussurea subulata*, *Rhodiola tangutic, Androsace tapete*, *Arenaria kansuensis*, *Astragalus* sp., *Oxytropsis* sp., *Leontopodim pusillum*, *Saussurea gnaphlodes* and *Rumex alpina*.

Sampling work was done in August, the warmest month of a year in Qinghai–Tibet plateau, in 2002. Among them, Drill I and II were located in the south side of Wudaoliang, in which mean annual air temperature was about –5.5 to –6.5°C, mean annual ground temperature was about –1.0 to –1.8°C (Wu and Liu 2004). Drill I and II had a depth of 570 and 485 cm, respectively. In order to know the age of permafrost soil, two samples (1.7 and 5.8 m in depth away from surface) were chosen for radioactive ^{14}C determination with the procedure described by Zhu et al. (2001). The age was about 22,680 ± 179 years old in the depth of 1.7 m, and 32,342 ± 430 years old in 5.8 m.

The drills, from surface to bottom, were made up of humus, sand soil, clay, gray muddy soil (including ground ice), and well weathered muddy sandy stone. In addition, six surface samples were collected at a depth of 20 cm along with Qinghai–Tibet Highway. Totally, 18 soil samples, 11 of them from active layer and 7 from permafrost, were utilized (Table 1). The mean annual temperature ranged from –2.21 to 2.22°C (Table 1).

To avoid contamination of organisms nonindigenous, the samples were collected according to the precious method (Khlebnikova et al. 1990; Shi et al. 1997; Vishnivetskay et al. 2000). Briefly, the soil cores of 15 cm in diameter were obtained by drilling using the column rotation method without washing drilling fluid. Samples were taken at intervals carefully, shaved the surface layer of core with a sterilized knife and put this core into a aseptic aluminium tin, sealed, then quickly kept it in refrigerating box under –18°C till processing in the lab.

Table 1 Sampling sites and physicochemical character of Qinghai–Tibet Plateau permafrost

No.	Depth (cm)	Lat. (N)	Long. (E)	Alt. (m)	Temp. (°C)[a]	Water content (%)	PH	Conductivity (mS/cm)	Organic compound (g/kg)	Nitrogen content (g/kg)
S1	20[b]	32°24'	91°43'	4804	2.01 ± 8.94	Not analyzed				
S2	20[b]	35°01'	93°00'	4628	2.21 ± 7.62	9.26	7.92	10.08	2.21	0.21
S6	20[b]	35°22'	93°26'	4527	2.21 ± 7.56	Not analyzed				
S7	20[b]	35°39'	94°04'	4807	2.13 ± 7.41	4.08	7.92	16.43	6.9	0.48
S9	20[b]	35°45'	94°20'	4125	2.16 ± 7.58	4.78	8.05	11.95	3.37	0.24
S10	20[b]	35°16'	93°08'	4631	2.20 ± 7.71	13.32	8.35	21.97	1.87	0.29
IB	30[b]	34°59'	92°59'	4621	2.19 ± 7.54	22.48	8.67	17.55	5.58	0.44
IF	100[b]	34°59'	92°59'	4621	–1.30 ± 5.09	12.29	8.18	12.69	2.28	0.44
IK	200[b]	34°59'	92°59'	4621	–1.97 ± 2.52	22.62	8.39	18.01	1.91	0.41
IT	360[c]	34°59'	92°59'	4621	–2.19 ± 0.84	24.28	8.91	1.08	2.41	0.55
IS	380 [c]	34°59'	92°59'	4621	–2.20 ± 0.74	24.28	8.72	8.64	1.91	0.37
IU	400[c]	34°59'	92°59'	4621	–2.21 ± 0.63	29.64	9.03	8.86	1.58	0.36
IX	570[c]	34°59'	92°59'	4621	–2.21 ± 0.21	12.28	8.72	1.72	2.46	0.63
IIA	20[b]	34°50'	92°56'	4676	2.22 ± 7.70	11.55	8.22	12.89	15.91	0.84
IIK	190[b]	34°50'	92°56'	4676	–1.78 ± 1.94	Not analyzed				
IIS	360[b]	34°50'	92°56'	4676	–2.02 ± 0.70	27.51	8.03	4.41	6.68	1.09
IIV	430[b]	34°50'	92°56'	4676	–2.03 ± 0.48	35.68	8.29	3.82	6.54	0.84
IIX	485[b]	34°50'	92°56'	4676	–2.01 ± 0.37	37.62	8.54	4.11	4.59	0.37

[a] The mean annual temperature in situ of Qingha–Tibet Plateau permafrost. The data were showed as mean annual temperature ± standard deviation

[b] Active layer

[c] Permafrost layer

Determining chemical characteristics of samples

Soil pH was measured using a soil/water (1:1 w/v) with a portable acidity meter (PHBJ-260, Shanghai Rex Instrument Factory, China). Air-dried soils were passed through a 100-mesh screen, then soil organic matters and total *N* determined with an element analyser (Perkin Elmer 2400 II CHNS/O analyser).

Culture media and isolation procedure

To optimize the isolation, low-nutrient medium, modified PYGV, was utilized in combination with a low-temperature incubation condition (Shi et al. 1997; Rivkina et al. 2000; Vishnivetskay et al. 2000). The medium PYGV (Staley 1968) was utilized to isolate alkaliphiles with modification by adding $NaHCO_3$ at a final concentration of 100 mM. After autoclave, the pH of the medium was round 9.

Sediment samples were diluted by aseptically weighing 5 g of wet sediment into a 250-ml flask containing 45 ml 0.85% NaCl solution with glass beads, and shaking with 150 rpm for 30 min at 4°C. Serial dilutions were plated on prechilled 1× concentrated modified PYGV agar (MPA) and incubated aerobically at 4°C. A sediment sample that had been autoclaved twice was utilized in the culture procedure as a negative control. Up to 30 days, the pH of the medium was tested again and the colonies were counted. Distinct colony types on the spread plates were purified by streaking and restreaking on MPA and then preserved at –70°C in liquid modified PYGV media (LMP) with 15% glycerol immediately.

Phenotypic traits analysis

After purification, fresh colonies from the 2× concentrated MPA incubated at 19°C for 48 h were prepared for phenotypic traits analysis. Morphological characteristics were examined by optical microscope. Gram stain status was determined by lysis with 0.2 M KOH (Manafi and Kneifel 1990) and validated with standard Gram stain. The ability to produce extracellular protease or amylase was detected following the methods described by Sánchez-Porro et al. (2003). Extracellular cellulase producers were screened out as described by Teather and Wood (1982).

The temperature, pH and NaCl concentration range for growth

The representative strains, which were also selected for 16S rRNA gene sequence determination, were employed to determine the temperature, pH and NaCl concentration range for growth. To determining the temperature and pH range for growth, cell suspensions

of each strains were prepared at logarithmic phase in 2× concentrated LMP.

The temperature range was tested in 2× concentrated LMP (pH = 9) with inoculating the identical volume of cell suspensions and incubated at 4, 6, 13, 19, 28 and 37°C. Up to 7 days, the growth status was monitored by optical densities at 540 nm. The growth rates of strain Tibet-IBa2 were examined with time elapsing after incubating at 4, 13 and 19°C.

The pH range for growth was determined in the 2× concentrated LMP (without $NaHCO_3$ before adjusting pH), the pH being adjusted to 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.5 using buffer solution prepared according to Gomori (1955). The final concentration of the series buffer was settled at 100 mM and the pH of the series media were verified after autoclave. Optical densities at 540 nm was monitored after growing in a shaker at 19°C for 24 h.

The NaCl concentration range was determined by growing cells in 1× concentrated MPA (pH = 9) that was added varying amounts of NaCl at 0, 5, 10, 15, 20 and 25%.

Amplified ribosomal DNA restriction analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) was utilized to group the isolates. The 16S rRNA genes were amplified with 8F (5′-AgAgTTTgATCCTggCTCAg-3′) and 1492R (5′-ggTTACCTTgTTACgACTT-3′) (Lane 1991). Genomic DNA was extracted and purified as described by Sambrook et al. (1989). Amplification reactions were performed in a total volume of 50 ul containing 2 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA), 1 × *Taq* buffer, 2 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, 10 pmol primer each and approximate 20 ng template DNA. All reaction mixtures were incubated in a thermal cycler (Applied Biosystems GeneAmp PCR System 2700) for 2 min at 94°C and then subjected to 30 amplification cycles of 1 min at 94°C, 58°C, and 1.5 min at 72°C, followed by a final extension step of 10 min at 72°C.

A 5 µl aliquot of each PCR mixture containing approximately 1.5 µg of amplified 16S rDNA was digested with 1.5 U of restriction enzyme *Hae*III and *Hin*6I (MBI Fermentas) in a total volume of 20 µl at 37°C for 16 h. The enzyme was inactivated by heating the preparations at 65°C for 15 min, and the reaction products were separated on a 2.5% gel (wt/vol) electrophoresis by using a 100-bp ladder as a marker in TAE buffer containing 0.5 µg of ethidium bromide per ml.

Sequence and phylogenetic analysis

Based on amplified ribosomal DNA restriction analysis (ARDRA) of isolates, one representative strain of each group was selected for 16S rRNA gene squence determination. Four primers were utilized for sequencing as they were 27F, 5′-AgAgTTTgATCCTggCTCAg-3′ (*Escherichia coli* positions 8–27), 338R, 5′-gCTgCCTCCCgTAggAgT-3′ (337–353), 517F, 5′-CCAgCAgCCgCggTAAT-3′ (517–533) and 907F, 5′-AAACTCAAATgAATTgACggg-3′ (906–926) (Lane 1991). Almost-complete 16S rDNA nucleotide sequences were determined.

The 16S rRNA gene sequences were aligned against representative reference sequences of the most closely related members, obtained from the Ribosomal Database Project (Cole et al. 2003) and the European Molecular Biology Laboratory (EMBL), by use of the multiple-alignment CLUSTALW 1.81 software package (Thompson et al. 1997). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances, phylogenetic dendrograms were constructed by the neighbor-joining method (Saitou and Nei 1987), and tree topologies were evaluated by performing bootstrap analysis (Felsenstein 1985) of 1,000 data sets by use of the MEGA2 package (Kumar et al. 2001).

Nucleotide sequence accession numbers

The 16S rDNA sequences of the 12 representative isolated strains have been deposited in the GenBank under accession numbers DQ108394-DQ108405.

Results

Physicochemical characterization of soil samples

The pH of the soil samples was among 7.92 and 9.03 (Table 1). Obviously, they were alkaline. The conductivity ranged from 1.08 to 21.97 mS/cm and the average was 10.28 ± 6.02. The water content was between 4.1 and 49.9% with the arithmetic mean at 21.8 ± 12.9%. The samples from Drill II contained more water than the samples from Drill I. The water content of the surface samples had minimum in average and changed most among the sample sites. Total aqueous ion varied between 0.38 and 9.02 mg Eq/100 g and the average was 2.41 ± 2.31. The average content of organic compound in each kilogram dry soil was 4.41 ± 3.49 g with the maximum at 15.91 and the minimum at 1.58. Each kilogram dry soil contained 0.21–1.09 g total nitrogen. The above data indicated

that except for pH, the physicochemical values of the samples were highly variable, and did not correlate with site location or depth.

The abundance and diversity of the viable bacterial population

Figure 1 showed the quantities and the diversity of the viable bacterial recovered from the 18 samples. After incubating at 4°C for 30 days, the quantities of colony forming unit on MPA were varied between 2.64×10^2 and 4.96×10^5 CFU/g of dry soil. Totally, 48 individual strains were selected according to morphological characteristics. The numbers of the individual strains in different samples were ranged from 1 to 6 (Fig. 1). Those samples from Drill II not only had higher diversity but also yielded more CFUs than the samples from Drill I or surface samples (Fig. 1). In the samples of Drill I, except IX (570 cm), the quantities of CFUs decreased with increasing the depth of the samples (Fig. 1).

ARDRA and phylogenetic analyses

Through amplified ribosomal DNA restriction analysis (ARDRA), all 48 isolates were clustered into 12 groups. After comparing 16S rDNA sequence of the representative strain of each group, the recovered population fell into three categories: high G + C gram positive bacteria, low G + C gram positive bacteria, and gram negative α-proteobacteria (Fig. 2).

The recovered population was dominated by high G + C gram-positive bacteria (Fig. 1). Twenty-eight isolates were belonged to this category and subdivided into three genera: *Nesterenkonia*, *Arthrobacter* and *Citricoccus* (Fig. 2). The group represented by Tibet-IBa2 was the most widely distributed and the most frequently isolated strains in the Qinghai–Tibet Plateau permafrost region. They were phylogenetically related to the species *Nesterenkonia lutea*. Another group represented by Tibet-IIVa3, the second most widely distributed and frequently isolated strains, was related to the species *Arthrobacter agilis*. The other two groups represented by Tibet-ITa1 and Tibet-ITa3, had high identities with the species *Arthrobacter sulfurous* and *Citricoccus alkalitolerans*, respectively. Counted the four groups together, 82.3% of the total emergence strains were belonged to this category (Fig. 2).

Six strains that represented 17 rare isolates were low-G + C gram-positive bacteria, namely related to different species of the genera *Marinibacillus*, *Sporosarcina*, *Planomicrobium* and *Bacillus* (Fig. 2). In all, this category shared 7.2% of all the emergence strains (Fig. 1). Here, stains Tibet-II Va1 and Tibet-II Va2 were both mostly related to species *Planomicrobium mcmeekinii* while the calculated similarities of 16S rDNA sequence for were 99.6 and 97.8%, respectively.

Only two strains represented three isolates were gram negative. They were both in the class of *α-proteobacteria* and strongly related to the genera *Mycoplana* and *Paracoccus*. The strain Tibet-IBa1 had 99.7% identity to *Mycoplana bullata*. The strain Tibet-S9a3 was related to *Paracoccus zeaxanthinifaciens* with an identity of 99.1%. The isolates in this category were only three but they all got high quantity colony when emerged (Fig. 1). Thus, this category shared 10.5% of all the emergence strains.

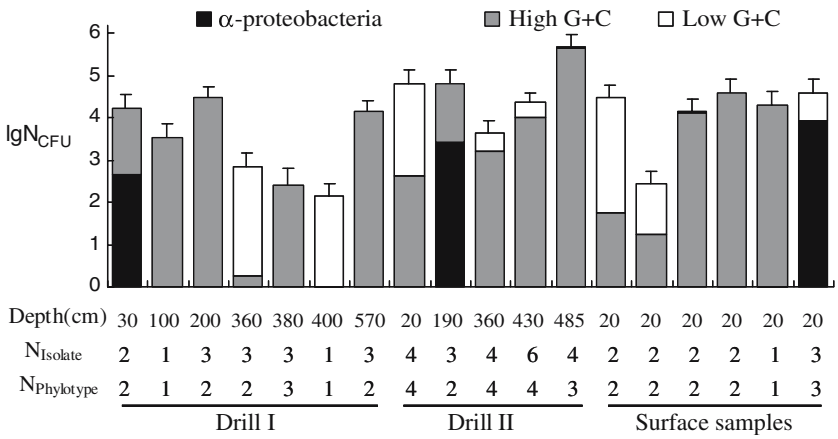

Fig. 1 The distribution and abundance of the populations were displayed as percentage and logarithm of the CFU number from the Qinghai–Tibet Plateau permafrost region. The depth of each sample was showed below the histograms. $N_{Isolate}$ indicated the quantities of the individual strains isolated from the corresponding samples and $N_{Phylotype}$ indicated the quantities of the ARDRA patterns of those isolates from the same samples

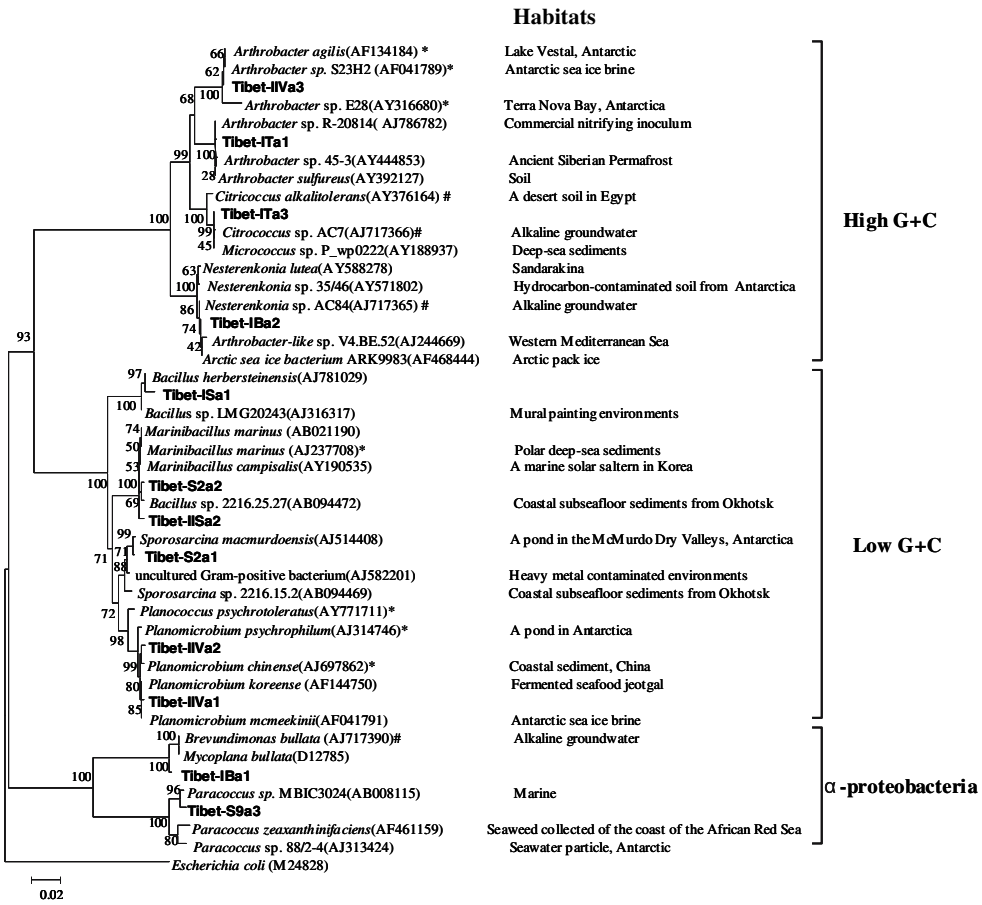

Fig. 2 Phylogenetic dendrogram based on a comparison of the 16S ribosomal DNA sequences of the 12 representative isolates from the Qinghai–Tibet Plateau permafrost region and some of their closest phylogenetic relatives. The tree created by the neighbor-joining method. The *numbers* on the tree indicate the percentages of bootstrap sampling derived from 1,000 replications. The presentative isolates characterized in this study are indicated in bold. *Bar*, 2 inferred nucleotide substitutions per 100 nucleotides. *Habitats* listed the environments where the organisms or sequence retrieved. *Shows psychrophlic or psychrotolerant bacteria, and # indicates alkaliphilic or alkalitolerant bacteria

Phenotypic traits analysis

Some phenotypic traits of 12 strains were listed in Table 2. The isolated population was dominated by gram positive with a majority of rod bacteria and a few cocci (Table 2). The colony colors changed from white to yellow.

Largely, the isolates could grow with NaCl concentration below 15% (Table 2), namely nonhalophilic. Within 12 representative strains, half of them produce extracellular protease, one-third produce extracellular amylase and 25% strains produce extracellular cellulase (Table 2).

Table 2 Phenotypic traits of representative strains of the isolate populations from permafrost of Qinghai–Tibet Plateau

Strain	Colony pigmentation	Cell morphology	Gram stain	Extracellular hydrolytic enzymes	NaCl range[a] (%)
IBa1	Milky	Short rod	–	Protease	0–5
IBa2	Yellow	Short rod	+	Protease, Cellulase	0–15
ISa1	White	Large rod	+	Amylase, Protease	0–20
ITa1	Milky	Short rod	+	–	0–15
ITa3	White-yellow	Short rod	+	Protease	0–15
II Sa2	Red-brown	Short rod	+	Amylase	0–15
II Va1	Orange	cocci	+	Protease	0–10
II Va2	Dark yellow	cocci	+	Protease, Cellulase	0–10
II Va3	Yellow	Short rod	+	–	0–15
S1a2	Beige	Short rod	+	Amylase, Cellulase	0–5
S2a2	White	Short rod	+	Amylase	0–15
S9a3	Dirty white	Short rod	–	–	0–20

[a] The isolates can growth within the range of NaCl concentration

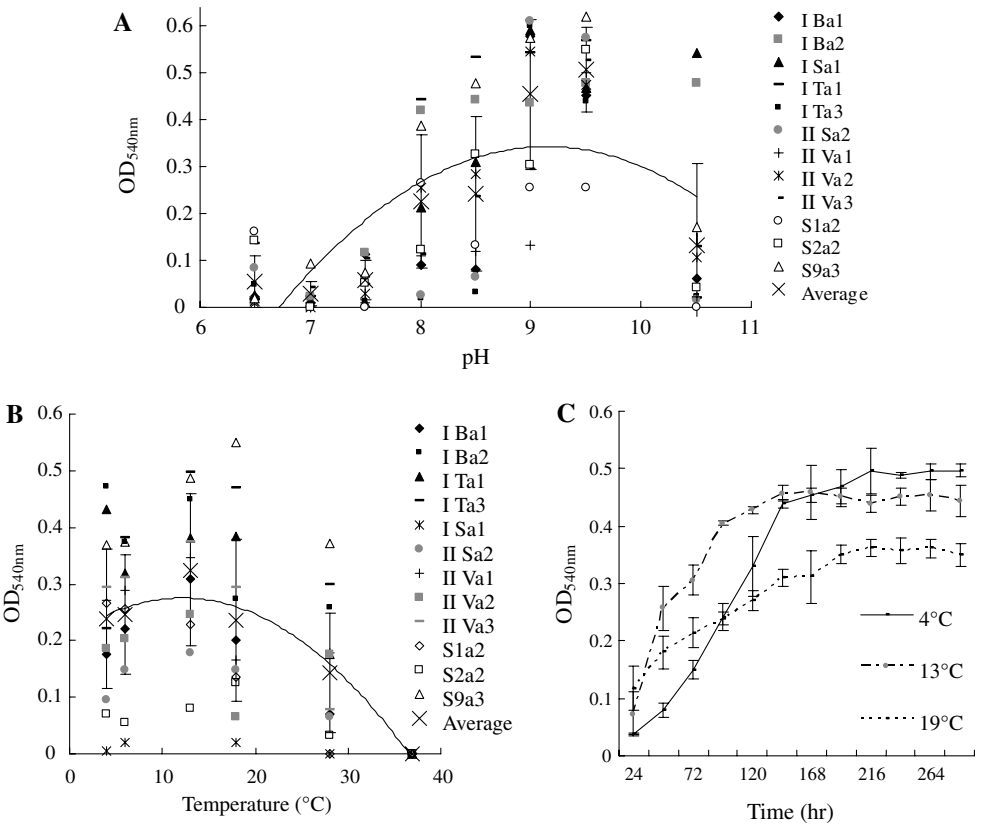

Fig. 3 The pH and temperature range for recovered population from the Qinghai–Tibet Plateau permafrost region. *Graph A* indicated the pH range for growth of the recovered populations. The abscissa was pH value. The data along *y*-axis indicate turbidity values. *Graph B* showed the temperature range for growth of the recovered populations. The abscissa and *y*-axis were evaluated with temperature and turbidity at OD_{540}, respectively. In *graph A* and *graph B*, the arithmetic mean (average) was used to engender trendline with standard deviation as error bar. *Graph C* illustrated the growth graph of the strain Tibet-IBa2 at 4, 13 and 19°C. The abscissa and *y*-axis were evaluated with time and turbidity

Figure 3A indicated the pH range for growth of the population in three graphs. The isolates mainly grew between 6.5 and 10.5. Except strain Tibet-ITa1 that grew well between 6.5 and 9, the other strains had an optimal pH between 9 and 9.5 (Fig. 3a).

The temperature for the recovered populations to grow was below 37°C. The population grew well below 20°C and had an optimal range between 10 and 15°C (Fig. 3b). Figure 3c show the growth rates at different temperatures of the strain Tibet-IBa2, the most widely distributed and the most frequently isolated strains in the Qinghai–Tibet Plateau permafrost region. In the first 24 h, Tibet-IBa2 grew fastest at 19°C and grew slowest at 4°C. However, Tibet-IBa2 grew better at lower temperature and got a highest final cell density at 4°C.

Discussion

The Qinghai–Tibet plateau represents a unique permafrost environment, being a result of very high elevation in a low latitude region caused by land uplift (Wang and French 1995). The permafrost of the Qinghai–Tibet Plateau was youngest and highest in the world (Cheng 1998). In addition, the permafrost of the Qinghai–Tibet Plateau was warmer than the higher latitude permafrost (Gilichinsky and Wagener 1995; Vorobyova et al. 1997). These unique traits suggested that the Qinghai–Tibet Plateau permafrost region provide a specific ecological niche and some special microbial lineages could be gained in the Qinghai–Tibet Plateau permafrost region.

In the study, the investigations were focused on alkaliphilic psychrotolerant bacteria in the Qinghai–Tibet Plateau permafrost region because of the prevailing alkaline and low temperature conditions. As Tiago et al. (2004) concluded, alkaliphilic bacteria are not confined to a single group or to a defined set of phylogenetic lineages; instead, they are distributed in various evolutionary branches (Jones et al. 1994, 1998; Krulwich 2000). Thus, molecular methods based on direct extract community DNA from soil were weakly to assess the diversity of alkaliphilic bacteria. In addition, due to low biomass and much inhibitors in permafrost, there were only few reported works investigated permafrost-preserved DNA (Willerslev et al. 2003; Lydolph et al. 2005). Furthermore, 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. Some 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. The diversity and distribution of alkaliphiles from the Qinghai–Tibet Plateau permafrost region were investigated based on culture procedure.

To our knowledge, the population of alkaliphilic psychrotolerant bacteria in the Qinghai–Tibet Plateau permafrost region was isolated firstly in our study. The abundance of alkaliphilic bacteria was varied between 10^2 and 10^5 cells g^{-1} of dry soil in the present study that show good coincident to the statistical frequency of alkaliphilic microorganisms in an environment of pH around 8 (Horikoshi 1999). Generally, viable bacteria abundance decreased with the increasing permafrost age (Friedmann 1994; Shi et al. 1997; Vishnivetskay et al. 2000). However, in this study, the phenomenon was not obvious for the viable alkaliphilic bacteria. In Drill I, the quantities of CFUs decreased, with an exception IX, when the depth of the samples increased (Fig. 1). In Drill II, the bacteria abundance showed no correlation with the depth. It was suggested that many factors, such as pH, temperature and composition of the sample, were in combined with age to influence the viable alkaliphilic bacteria abundance. For example, the samples from Drill II contained more water than the samples from Drill I or surface samples. This factor might contribute to the samples from Drill II yielded more CFUs than the samples from Drill I or surface samples (Fig. 1).

The isolates in present study were composed of 82.3% high G + C gram positive bacteria dominated with *Micrococcaceae* family, 7.2% low G + C gram positive bacteria, and 10.5% gram negative α-proteobacteria. The community composition was a little bit different from Shi et al. (1997), who reported viable bacteria of Siberian permafrost phylogenetically fell into four categories: 31.0% high G + C gram positive bacteria, 34.5% low G + C gram positive bacteria, 20.7% β-proteobacteria and 13.8% γ-proteobacteria. These differences may attribute to distinguished formation of permafrost (Gilichinsky 2002), distinctive physio-chemical characters of soil samples (Gilichinsky and Wagener 1995), different pH of culture medium and different temperature in isolation procedures (Shi et al. 1997; Vishnivetskaya et al. 2000).

In phylogenetic analysis, we found there were such similarities between the representative strains and some most closely related members as strains that came from alkalic or cold environments (Fig. 2). For example, in a heterotrophic aerobic populations recovered from an alkaline groundwater, Tiago et al. (2004) isolated two strain named *Nesterenkonia* sp. AC84 and *Citrococcus* sp. AC7 had a calculated similarity of 16S rDNA qequence of 99.86 and 99.72% with Tibet-IBa2 and Tibet-ITa3, respectively. In addition, the majority of the recovered population from the alkaline groundwater and from Qinghai–Tibet Plateau permafrost had a same phylogenetic ascription. And also, the negative bacteria were both rare in the two environments (Tiago et al. 2004).

In our study, many strains isolated from the Qinghai–Tibet Plateau permafrost region had high phylogenetic similarity to psychrophilic or psychrotolerant bacteria from cold environments, such as ancient Siberian permafrost, ponds in Antarctica, antarctic sea ice brine and polar deep-sea sediments (Junge et al. 1998; Michaud et al. 2004; Reddy et al. 2002; Ruger et al. 2000). Though all the isolates showed psychrotolerant ability, no true psychrophiles were found this time, which was consistent with a common and significant phenomenon that psychrophiles were absent or very rare in terrestrial habitats (Vorobyova et al. 1997; Vishnivetskay et al. 2000).

Extremophiles had a wide application in industry and biotechnology because there were always useful alkaline enzymes and metabolites (Ito et al. 1998; Rothschild and Mancinelli 2001). We tested the activity of extracellular enzymes production of all isolates and found that many of the isolates could excrete protease, amylase and cellulase (Table 2), which was consistent with the microorganisms isolated from Siberia permafrost (Tiedje et al. 1994). The preparatory results aroused that the further works, such as to characterize the enzymes and to establish their novelty, was needed for exploitation some potential indeustrial application of alkaliphiles in the Qinghai–Tibet Plateau permafrost region.

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