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ORIGINAL PAPER

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Bacterial diversity of the Inner Mongolian Baer Soda Lake as revealed by 16S rRNA gene sequence analyses

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Abstract Bacterial diversity associated with Baer Soda Lake in Inner Mongolia of China was investigated using a culture-independent method. Bacterial 16S rRNA gene libraries were generated using bacterial oligonucleotide primers, and 16S rRNA gene sequences of 58 clones were analyzed phylogenetically. The library was dominated by 16S rDNAs of Gram-negative bacteria (24% α-Proteobacteria, 31% β-Proteobacteria, 33% γ-Proteobacteria, and 2% δ-Proteobacteria), with a lower percentage of clones corresponding to Gram-positive bacteria. Forty cloned sequences were similar to that of known bacterial isolates (>97% sequence similarity), represented by the species of the genera *Brevundimonas*, *Comamonas*, *Alcaligenes*, *Stenotrophomonas*, and *Klebsiella*. Eighteen cloned sequences showed less affiliation with known taxa (<97% sequence similarity) and may represent novel taxa.

Keywords 16S rRNA gene · Alkaliphilic bacteria · Biodiversity · Phylogenetic analysis · Soda lake

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Introduction

Soda lakes have very high productivity and show active decomposition processes (Grant et al. 1990; Zhilina and Zavarzin 1994). To gain insight into the structure and function of the microbial communities, it is first necessary to characterize the microbiological inhabitants of soda lakes. Several authors have isolated a number of alkaliphiles from soda lakes that showed considerable phylogenetic diversity. Physiological types include proteolytic, cellulolytic, saccharolytic, acetogenic, and sulfate-reducing bacteria and methanogenic and halophilic archaea (Zavarzin et al. 1999; Jones et al. 1998; Duckworth et al. 1996; Zhilina and Zavarzin 1994). Nevertheless, due to the difficulty in the cultivation of many organisms and to the limitations of culture-based methods mainly for specific physiological groups of microorganisms, cultured microorganisms may represent minor components of the existing diversity (Torsvik et al. 1990; Amann et al. 1995).

The development of molecular biological techniques that do not depend upon culture has proven effective for gaining insight into the diversity and structure of microbial communities. In particular, the ability to recover and analyze 16S rRNA genes directly from environmental DNA provides a means to investigate microbial populations in any environment without the need for culture, eliminating the absolute dependence on the isolation of pure cultures (Olsen et al. 1986; Ward et al. 1990; Amann et al. 1995; Hugenholtz et al. 1998; Dojka et al. 2000). As far as soda lake are concerned, archaeal communities have been analyzed by the direct 16S rRNA gene cloning of environmental DNA, and this analysis has revealed the presence of two distinct new archaeal lineages (Grant et al. 1998). However, to date, culture-independent molecular phylogenetic techniques have not yet been applied to analyze bacterial diversity in soda lakes.

Baer Soda Lake is located in the Autonomous Region of Inner Mongolia in China. Its microbial diversity based on culture procedures has been described

previously (Zhang et al. 2001). In order to further explore its microbial community, we investigated its phylogenetic diversity using PCR amplification and cloning of bacterial 16S rRNA genes directly from DNA samples. Here, we report the results of the phylogenetic analysis of the cloned 16S rRNA genes, which show a rich diversity. Although soda lakes are well known for their bacterial diversity, this report represents the first description of the bacterial diversity investigated using culture-independent molecular phylogenetic techniques.

Materials and methods

Sampling

Baer Soda Lake is located in the Hulunbeir area of Inner Mongolia, Autonomous Region of China, at 49°24′ N and 118°08′ E. The lake is shallow, with 0.1–0.15 m depth and cover area of approximately 1 km^2. Samples of the upper 1–10 mm of sediment were collected in a sterile plastic bag, transported at ambient temperature to our laboratory in Beijing, and stored at 4°C until analysis. The sample pH was 10 and mineralization was 82 g/l.

Extraction and purification of total genomic DNA

Isolation of DNA from the sediment sample followed the method described by Tsai and Olson (1991). Some minor modifications were introduced: 10 g sediment sample was desalted by dialysis against TE buffer (pH 8) before treatments with lysozyme and freeze-thaw cycles; extractions with phenol and with chloroform were followed by treatment of the aqueous phase with polyvinylpolypyrrolidone (PVPP) (Sigma) before precipitation and washing of DNA. The crude DNA obtained was electrophoresed on 1% agarose gels and purified by electroelution into dialysis (Sambrook et al. 1989).

Amplification, cloning, and sequencing of 16S rDNA

16S rRNA genes were amplified from the total DNA extracted as described here by PCR with two bacterial primers: 27f (5′-GAGA GTTTGATCCTGGCTCAG; positions 7–27 in the 16S rRNA of *Escherichia coli*) and 765r (5′-CTGTTTGCTCCCCACGCTTTC; positions 765–785 in the 16S rRNA of *E. coli*) (Brosius et al. 1978). The reaction mixtures contained 1.5 mM $MgCl_2$, 0.2 M each dNTP, 25 pM each primer, 100 ng template, and 2.5 U *Taq* DNA polymerase (Promega, Madison, Wis., USA) with a reaction buffer supplied by the manufacturer in a total volume of 100 µl. A hot start of PCR was performed at 98°C 5 min prior to addition of the *Taq* DNA polymerase. Thirty cycles of 45 s at 95°C, 45 s at 45°C, and 90 s at 72°C, with a final 10-min extension at 72°C, followed.

The PCR products were purified using the glass-milk clean-up kit (BioDev, China) and then cloned to the pGEM-T vector using T-Easy System kit (Promega, Madison, Wis., USA), following the manufacturer's protocol. White clones were checked for size of insert by the alkaline-SDS rapid colony disruption method (Sambrook et al. 1989). The nucleotide sequences of plasmid inserts selected randomly were determined on the Applied Biosystem model 377 DNA sequencer (Foster City, Calif., USA).

Phylogenetic analysis

The sequences were compared to 16S rRNA gene sequences available in the GenBank databases by the BLAST search. All sequences were checked for possible chimeric artifacts by the CHECK_CHIMERA program in the Ribosomal Database Project (RDP) (Maidak et al. 1999). Multiple sequence alignments were performed using ClustalW version 1.8 (Thompson et al. 1994). The similarity percentages were calculated by the method of Jukes and Cantor (1969) in the ClustalW program. The phylogenetic analysis of multiple sequence alignments was performed with TreeconW version1.3b (Van de Peer and De Wachter 1994). Phylogenetic tree construction was carried out by the neighbor-joining method (Saitou and Nei 1987) with the Kimura two-parameter calculation model in TreeconW v.1.3b. Representative sequences have been assigned GenBank accession numbers AF506988–AF50015.

Results and discussion

To assess the diversity of bacteria in Baer Soda Lake, the total genomic DNAs were extracted directly from Baer Soda Lake sediment sample and were amplified using the bacterial 16S rDNA primers. To avoid a possible bias occurring during PCR amplification (Liesack et al. 1991; Suzuki and Giovannoni 1996), an approximately 700-bp fragment from the 5′ terminus (27–765 region *Escherichia coli* numbering) was amplified. This section contains variable and conserved regions, which could reflect well the phylogenetic position of the corresponding 16S rDNA sequences and the degree of diversity in a clone library (Schmidt et al. 1991; Bond et al. 1995; Stackebrandt and Rainey 1995). The 60 clones (designated Br-), which contained partial 16S rRNA genes, were sequenced. Two apparent chimeric sequences were omitted from further studies. The 16S rRNA gene sequences of the 58 clones with an average length of 700 nucleotides (minimum, 560 nucleotides; maximum, 780 nucleotides) were compared to the 16S rDNA databases, and nearest-neighbor sequences were identified. Table 1 shows the similarities between representatives of clone groups and the sequence of a described species or, in some cases, the sequence of uncultured clone. The phylogenetic analyses of the partial 16S rRNA gene sequences of the 58 clones indicated that the majority of these clones were related to the class Proteobacteria, with only six clones affiliated with Gram-positive bacteria.

As shown in Fig. 1, the 52 cloned sequences that belong to the Proteobacteria clustered into four of the five classical subdivisions—α, β, γ, and δ— as follows. Fourteen clones appear to belong to the α-subdivision of Proteobacteria. Of these, seven clones (represented by Br-33) showed a high similarity to the 16S rDNA of *Brevundimonas vesicularis* (97% or higher sequence identity, Abraham et al. 1999), so that they probably belong to this or a similar taxon. In contrast, the other seven clones (represented by Br-20) were closely related to each other (97% or higher sequence identity) and formed a single cluster distantly related to known sequences in the α-Proteobacteria. They had less than 93% sequence similarity to known taxa (Table 1) and may represent a novel taxon. Eighteen clones were affiliated with the β-subdivision of Proteobacteria. All but three of the clones belonged to the *Comamonas* cluster: 14 of the clones (represented by Br-13) seem to

Table 1 Phylogenetic affiliation of partial 16S rDNA clone sequences obtained from sediment of Baer Soda Lake

Division	Representative clone of clone group	No. of total clones[a]	Nearest phylogenetic neighbor	Similarity (%) to nearest neighbor[b]
α-Proteobacteria	Br-33	7	*Brevundimonas vesicularis*	98.3–100
	Br-20	7	*Blastochloris sulfoviridis*	91.5–93.0
			Prosthecommicrobium pneumaticum	92.1–92.8
β-Proteobacteria	Br-z13	14	*Comamonas acidovorans*	97.8–100
	Br-z39	1	*Comamonas acidovorans*	96.0
	Br-54	3	*Alcaligenes fecalis*	99.5–99.8
γ-Proteobacteria	Br-z21	12	*Stenotrophomonas maltophilia*	97.8–99.9
	Br-23	4	*Klebsiella planticola*	97.1–99.5
	Br-z11	1	*Alkalispirillum mobile*	89.4
			Thioalcalovibrio denitrificans	89.4
	Br-45	2	*Halomonas campisalis*	94.6–95.6
δ-Proteobacteria	Br-14	1	*Desulfovibrio sulfodismutans*	82.7
Gram positive	Br-z3	2	*Bacillus carboniphilus*	95.2–95.7
	Br-15	1	Bacterium Chibacore	94.2
			Streptomyces lipmanii	87.1
Uncertain	Br-z22	1	Unidentified bacterium BD2-11	90.7
	Br-z33	1	Unidentified bacterium BD2-11	89.6
	Br-z19	1	Uncultured Antarctic clone bh6.6	89
			Deinococcus grandis	81.4

[a]Clones with more than 97% nucleotide identity were considered identical
[b]The range of similarities indicates the highest and lowest values of similarity to the nearest neighbor when more than one clone with over 97% identity was found

be a taxon similar to *Comamonas acidovorans* (97% or higher sequence identity, Anzai et al. 2000); the one remaining clone, Br-z39, showed 96% sequence similarity with *C. acidovorans* and may represent a new species of the genus *Comamonas*. Three clones (Br-z38, Br-54, and Br-z14) appear to be similar and share a taxon with *Alcaligenes fecalis* (more than 99.5% sequence identity, Schroll et al. 2001). Nineteen clones are clustered with γ-subdivision of Proteobacteria. Twelve of them (represented by Br-z21) belong to the similar taxon *Stenotrophomonas maltophilia* (97% or higher sequence similarity, Minkwitz and Berg 2001). Four clones (represented by Br-23) had *Klebsilla planicola* (Drancourt et al. 2001) as the nearest neighbor, with more than 97% sequence similarity. Two others (Br-50 and Br-45) have 97.7% sequence similarity to each other and 94.6% and 94.9% sequence similarity to *Halomonas campisalis* (Mormile et al. 1999), respectively, and may represent a new species of the genus *Halomonas*. The remaining one clone, Br-z11, had only low similarity to known sequences in the databases. The highest sequence similarity is 89.4% to *Alkalispirillum mobile* (Rijkenberg et al. 2001) and *Thioalcalovibrio denitrificans* (Sorokin et al. 2001), and this may represent a new taxon within the γ-subdivision of Proteobacteria. Only one clone (Br-14) seems to be affiliated with the δ-subdivision of Proteobacteria. It is distantly related to *Desulfonatronum lacustre* (82.7% sequence similarity, Friedrich 2002) and may represent a novel taxon within the δ-subdivision of Proteobacteria.

Figure 2 shows the phylogenetic relationships of Gram-positive bacteria and relatives. The six clones determined distribute among four groups and appear very atypical, although they occurred in low percentages of all clones. Two of them, Br-z3 and Br-z47, had 98.5% sequence similarity to each other and showed 95.2% and 95.7% sequence similarity, respectively, to *Bacillus carbonophilus* (Goto et al. 2000), indicating that they may represent a new species of the genus *Bacillus*. Br-15 showed 94% similarity to an invalidly published bacterium, Chibacore 1500 (only a sequence in the GenBank databases), and seems to be affiliated with the *Streptomyces* cluster (87.1% sequence identity to *Streptomyces lipmanii*). Br-z19 is individually deeply branched in the tree and cannot be affiliated with any known taxa. Br-z22 and Br-z33 showed 91.6% sequence similarity to each other and low similarity to cultivated strains and clustered with the unidentified bacterium BD2–11, which was retrieved from the deep sea. The last four clones may represent four novel taxa.

With the 16S rDNA phylogenetic analysis, we could characterize a number of diverse bacteria residing in Baer Soda Lake. Although some of our clones were related to alkaliphilic bacteria from soda lakes (e.g., *Alkalispirillum mobile*, *Thioalcalovibrio denitrificans*, and *Halomonas campisalis*), many of our clones were related to known species (more than 97% similarity) from non-alkaline environments. Examples are *Brevundimonas vesicularis* isolated from medical leech and stream (Abraham et al. 1999); *Comamonas acidovorans* from soils (Willems et al. 1991); *Alcaligenes fecalis* from freshwater and marine environments; and *Stenotrophomonas maltophilia* from clinical, Antarctic, and a wide range of other environments (Palleroni and Bradbury 1993). *Stenotrophomonas maltophilia* sequences have also been implicated as common laboratory contaminants, particularly under conditions where the starting environment has low biomass and therefore low levels of DNA (Tanner et al. 1998). However, the number of clones picked up here and the high nutrient and

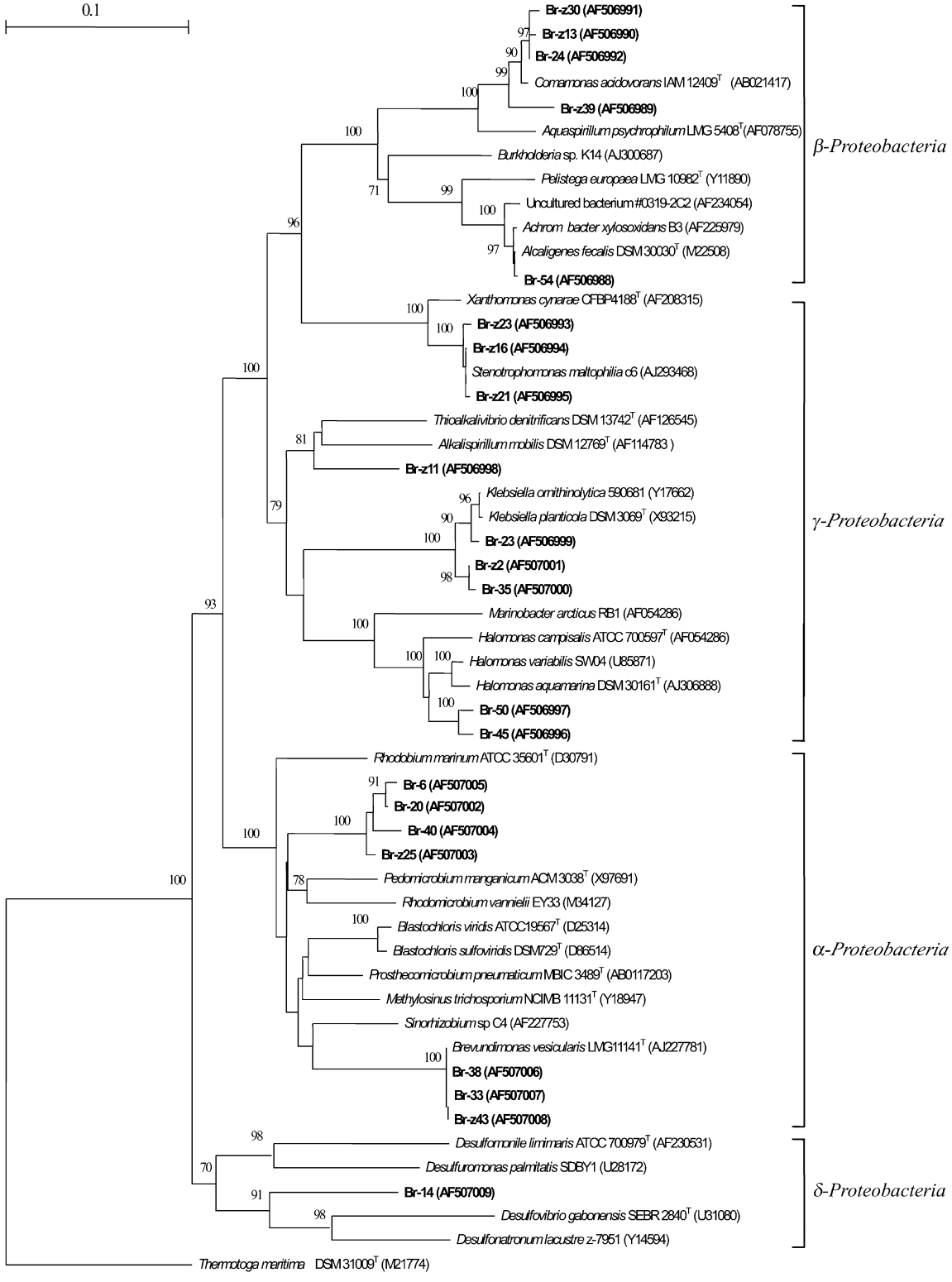

Fig. 1 Phylogenetic tree showing the relationship between the cloned 16S rDNA sequences and those of taxa in the class Proteobacteria. Bootstrap values above 70% are shown. The *scale bar* represents the number of changes per nucleotide position. *Thermotoga maritima* served as the outgroup

therefore high biomass levels in soda lakes rule out any real possibility of these being contaminants. These results seem to imply that many strains with similar 16S rDNA sequences can thrive under very different physiological conditions and are widespread in nature, with likely alkaliphilic relatives populating soda lakes.

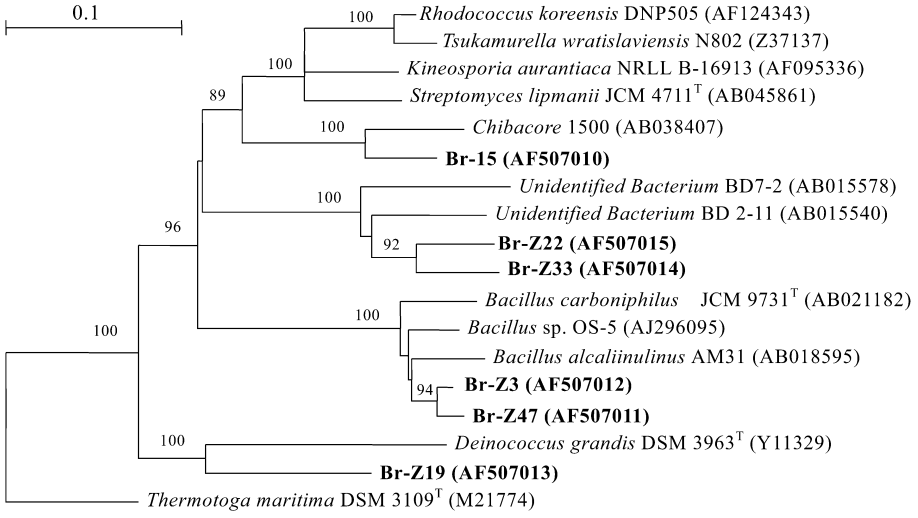

Fig. 2 Phylogenetic tree showing the relationship between the cloned 16S rDNA sequences and those of Gram-positive bacteria and relatives. Bootstrap values above 70% are shown. The *scale bar* represents the number of changes per nucleotide position. *Thermotoga maritima* served as the outgroup

Previous culture-based investigations of bacteria isolated from soda lakes have revealed great phylogenetic diversity, with microorganisms represented by the γ and δ subdivisions of the Proteobacteria; the Thermotogales, Gram-positive bacteria with high and low G+C content; the Spirochetes, sulfate reducers; and methanogenic and halophilic archaea. (Grant et al. 1990; Duckworth et al. 1996; Tourova et al. 1999; Zavarzin et al. 1999; Jones et al. 1998). Our results have extended the knowledge of the phylogenetic diversity of bacteria residing in soda lakes. The taxonomic positions of our clone sequences are affiliated with low and high G+C Gram-positive bacteria and Proteobacteria of the α, β, γ, and δ subdivisions. Among them, 18% of our clones were assigned to members of the α and β subdivision of Proteobacteria, which were not found previously among cultivated soda lake isolates. Even in the phylum containing isolates from soda lakes (such as the γ subdivision of Proteobacteria), the majority of the clones we selected were related to organisms not previously found in soda lakes. In addition, we have obtained some clones (such as Br-z19, Br-14, Br-z22, Br-15, and Br-20) that have low sequence identity to known sequences, and these may represent new taxa. These results suggest a rich diversity of bacteria present in soda lakes and that a large proportion of the Gram-negative microbial population from these sites has never been cultured.

The majority of our clones obtained from Baer Soda Lake appears to be phylogenetically affiliated with aerobic organotrophic bacteria. Baer Soda Lake is a shallow lake, and the sample was taken from the upper sediment of the lake. In this case the aerobic bacteria are likely to be a major part of the metabolically active community, as exemplified by the presence of phylotypes related to lipolytic and proteolytic bacteria (*Stenotrophomonas maltophilia* and *Alcaligenes faecalis*) and saccharolytic and organic acid-degrading bacteria (*Brevundimonas vesicularis* and *Comamonas acidovorans*). Clone types Br-33, Br-z13, and Br-z21 were the most common (7, 14, and 12 occurrences, respectively). Multiple detection of the identical sequences may also reflect that these corresponding aerobic bacteria were significant members of the communities that were probably active in the decomposition and mineralization of organic matter in Baer Soda Lake, although the number of 16S rDNA sequences does not show the good relationship with the number of corresponding bacteria due to the possible biases occurring during DNA extraction, PCR amplification, and cloning (Felske et al. 1997; Von-Wintzingerode et al. 1997; Burton and Norris 2000).

In the previous work performed in our lab, the biodiversity of Baer Soda Lake was investigated based on culture methods (Zhang et al. 2001). Fifty-three alkaliphilic bacteria were isolated from sediment samples, and 20 of them were subjected to 16S rDNA sequence analysis. These isolates were affiliated with the genera of *Bacillus*, *Amphibacillus*, *Gracilibacillus*, *Alkalibacterium*, *Salinicoccus*, *Exiguobactrium*, *Halomonas*, *Pseudomonas*, *Marinospirillum,* and *Cyclobacterium*. Of the 20 isolates, only 4 were Gram-negative, and Gram-positive isolates were diverse and predominant. However, the majority of the clones obtained from Baer Soda Lake were related to Proteobacteria, with only about 10% of the clones affiliated with Gram-positive bacteria. Comparing the 16S rDNA from isolates and clones, few of the cultivated isolates were related to the sequences of the clones obtained from Baer Soda Lake. Only *Halomonas* and *Bacillus* were detected with both the culture-independent approach and the cultivation method. Very different results were obtained by both approaches. This seems to be the case for many environments (Borneman and Triplett 1997; Gray and Herwig 1996). On the one hand, the results indicate that many bacterial cells in Baer Soda Lake are not yet cultured, and the culture-independent study may provide clues to obtain more bacteria in culture for studying their physiology and understanding their function in the community. On the other hand, our culture-independent study may expand our understanding of the biodiversity of Baer Soda Lake but is far from gaining the complete biodiversity.

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