Biotechnology Journal International

20(2): 1-16, 2017; Article no.BJI.36909
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

Prokaryotic Diversity of the Alkaline Lake Acıgöl, Turkey by Using Culture-dependent and Culture-Independent Methods

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Authors' contributions

This work was carried out in collaboration between all authors. Author GB designed the study, performed the experiments, and wrote the first draft of the manuscript. Author HHB helped for the design of the study, contributed to field study, managed the literature searches. Author EO contributed the experimental stages, managed the analyses of the study. Author BB contributed the experimental stages, managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2017/36909
Editor(s):
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Complete Peer review History: http://www.sciencedomain.org/review-history/22185

Original Research Article

Received 22nd September 2017
Accepted 30th November 2017
Published 7th December 2017

ABSTRACT

Aims: The prokaryotic diversity of Acıgöl lake, Turkey, was studied in samples taken from soil, water and mud. Culture -dependent and -independent methods were used to analyse the results.
Methodology: Bacterial cultures were isolated by using six different growth media. Isolates were identified according to their 16S *rDNA* sequence analysis. Liquid media were used to test antibacterial substance production of the isolates. *Bacillus cereus*, *Serratia marcescens*, *Pectobacterium carotovorum*, *Enterococcus faecalis*, *Listeria monocytogenes, Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Listeria innocua* were used as indicator bacteria.

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The extracted DNA samples taken from soil, water and mud were used in PCR reactions to amplify 16S *rDNA* genes by using universal bacterial primers. Amplicons were cloned by TA cloning after which white colonies were selected and colony PCR were applied for the amplification of the fragments. Each amplicon obtained from clones was sequenced and similarities were compared to those of the other bacterial groups. The DNA extracted from water sample was also used to amplify archeal 16S *rDNAs.* For the amplification reaction, the primers used were specific to the archaeal domain and their amplicons were cloned by TA cloning. Randomly picked white colonies were used for M13 PCR. The sequences of amplicons were analyzed by BLAST to determine the highest similarities with the other archeal groups.

Results: In this study, fourty-nine strains were identified as belonging to *Bacillus*, *Halomonas Enterococcus*, *Exiquobacterium*, *Piscibacillus*, *Haloalkalibacillus*, *Aerococcus*, *Acinetobacter*, *Lysinibacillus*, *Oceanobacillus*, *Planococcus*, *Micrococcus* genera according to 16S rDNA analysis. Among these isolates, the GBA17, GBA34 and GBA36 weakly inhibit the growth of *M. luteus.* The analysis of 16S *rDNA* from different environmental samples suggests that the bacterial clones belong to the class of Gemmatimonadetes (n=1), Alphaproteobacteria (n=4), Flavobacteria (n=1), Opitutae (n=2), Gammaproteobacteria (n=1), Saprospira (n=1), Verrucomicrobia (n=1). All of the identified archeal clones were affiliated to the Class Halobacteria with 12 members belonging to the Halobacteriales order and two members belonging to the Haloferaceles order. No match was found between the taxa determined by culture-dependent method and those determined by independent method. Growth media used in the isolation experiments might not support the growth of extreme halo-alkaliphilic bacteria found as a result of cloning.

Haloalkaliphilic and alkaliphilic isolates obtained in our study may be ideal candidates as industrially important substance producers.

Keywords: Lake Acigol; hypersaline; alkaline; prokaryotic diversity.

1. INTRODUCTION

Bacteria isolated from extreme environments are of great interest for microbiologists since they have extraordinary properties and have industrial potential. Optimal growth for alkaliphilic bacteria is greater than pH 9 and they have a great potential in terms of their various metabolites. Alkaliphiles can be isolated from samples like garden soil but, their numbers in alkaline environments are much higher. That's why soda lakes, which are an example of alkaline habitat, are a good source for alkaliphilic bacteria isolation [1].

Natural alkaline environments are not very common on earth. Soda lakes and soda deserts are the most studied of those habitats. These environments have a pH value of 10 on average, and can go up to 12. Soda lakes contain high levels of sodium carbonate and salt. They can also show hypersaline characteristics when the chlorine concentration is high [2]. Microbiologists who conduct research in those lakes are mainly focused on the isolation and characterization of organisms which have industrial potential and also the characterisation of the phylogenetic diversity of alkaliphilic-halophilic Bacteria and Archea.

Soda lakes in Rift valley in East Africa are the most studied among those extreme environments [3,4]. Mongolian Baer and Kenyan soda lakes are searched for biodiversity by using molecular methods [5,6]. The importance of the microorganisms in those lakes comes from the reminerilization capacity of their organic matter. Lonar Soda Lake in India [7], Soda Saline Crater Lake in Isabel Island, Mexico [8), and Central Asian soda lakes [9] are other examples in which archeal and bacterial diversity were studied in detail by traditional microbiological isolation methods or by molecular techniques. In the literature there are many studies related to the isolation and/or identification of microorganisms from alkaline habitats around the world but, research involving extremophile microorganisms in Turkey have yet to be investigated. Exploration of new isolates of alkaliphilic or haloalkaliphilic bacteria is needed for biotechnological and industrial aspects, primarily for the detergent and laundry industries. Enzymes, antibiotics and carotenoids have been reported as by-products from alkaliphiles [10]. Our aim in this research was (1) to isolate and identify halo-alkaliphilic microorganisms, (2) to determine their antibacterial activities and (3) to reveal the archeal and bacterial diversity by culture-dependent and -independent methods.

2. MATERIALS AND METHODS

2.1 Site Description and Sampling

Lake Acıgöl is located in the southwest of Turkey and is a hypersaline playa lake with salinity at about 7-7.5% and pH around 8.6 [11]. The estimated surface area of the lake is about 60 km^2 with a depth of 2–3 m [12]. Samples from mud, soil and water were taken in sterile glass and polyethylen bottles and stored at -20°C in the laboratory until the analysis.

2.2 Culture Medium and Isolation of Bacteria

Six different culture medium were used for the isolation of bacteria from three different environment samples. The recipes of the media used in this study are as follows:

Medium A (g/L): Glucose 10.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.2, Na_2CO_3 10.0, (agar 20.0) [13].

Medium B (g/L): Soluble starch 10.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.2, Na_2CO_3 10.0, (agar 20.0) [13].

Medium C (g/L): Peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, sodium chloride 5.0, (agar 20.0)., pH was adjusted to 10.0 with 1 N NaOH [13].

Medium D (g/L): Peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, sodium chloride 200.0, (agar, 20.0.) pH was adjusted to 10.0 with 1 N NaOH [13].

Medium E (g/L): Glucose 10.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.2, Na_2CO_3 10.0, NaCl 200, (agar 20.0) [14].

Medium F (g/L): Peptone 5.0, meat extract 3.0, KH_2PO_4 3.9, $MgSO_4.7H_2O$ 0.78, Na_2CO_3 21.4, $NaHCO_3$ 17, (agar 20.0) [15].

Samples of 1 g soil or mud or 1 mL water were incubated in 6 different medium conditions as described above and then one set of the tubes was incubated at 28°C and the others were incubated at 37°C. One week after the incubation had started, 100 µL of liquid cultures were transfered onto the agar media. The randomly picked colonies were purified twice and Gram stained. Pure culture of isolates were maintained in skimmed milk at -80°C.

2.3 16S *rDNA* Analyses and Phylogenetic Tree of Isolates

2.3.1 DNA extraction from isolates

The genomic DNA from bacterial isolates was extracted by using phenol-chloroform method according to [16]. Briefly, 1 mL of liquid culture was centrifuged at 4000 x g for 10 min at +4ºC, supernatant was decanted and 500 µL of lysis buffer was added to pellet, this mix was vortexed and centrifuged at 13000 rpm for 5 min. The supernatant was poured, and the pellet was mixed with 75 µL lysosyme (20 mg/ml) and 425 µL lysis buffer. The tube was inverted several times and incubated at 37ºC for 30 min. After incubation, 20 µL RNAse A (10 mg/mL) and 25 µL SDS (%20) were added to the mix and incubated again at 37ºC for 30 min. Then, 6,5 µL Proteinase K was added and the tube was incubated at 50ºC for 1 hour. Phenol-chloroform-isoamyl alcohol was added to the mix at equal volume (v/v) and then centrifuged at 13 000 rpm for 5 min. Clear phase was collected and added to a new tube and then mixed with Na-acetate (3 M, pH 5.5). Isopropanol (v/v) was added to the mix, and the tube was incubated at -20 C for 30 min and then centrifuged at 13 000 rpm for 20 min. The supernatant was decanted and 500 µL ethyl alcohol (70%) was added to pellet, and the tube was centrifugated at 15 000 rpm for 5 min. After the alcohol was poured and removed from the tube, the pellet was dissolved in 50 µL distilled water.

2.3.2 16S *rDNA* gene amplification

PCR was carried out to amplify 16S rDNA gene by using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction was set as follows; Taq buffer (1X), dNTP (200 µM), $MgCl_2$ (2 mM), primers (0,2 µM), Taq polymerase 1,25 U and 10 ng of the DNA as a template in 20 µL mix reaction. Reactions were run at Techne TC-3000 thermalcycler and the programme was set as follows: initial denature at 94°C for 5 min, 94°C for 30 sec, 50°C for 30 sec; 72°C for 10 sec for x35 cycles; and final extensions at 72°C for 15 min.

2.3.3 Sequencing and phylogenetic tree

PCR products of 16S *rDNA* gene were sequenced by using ABİ 3730 XL sequencer at MedSanTek (Turkey). The sequences were compared with those in GenBank using Basic Local Alignment Search Tool (BLAST) to determine the similarities. The number of bases compared with the indicator 16S rDNA sequences were between 471-582. Reference sequences were chosen based on BLAST similarities and used for phylogenetic tree with sequences obtained in our study. Sequences were aligned and a phylogenetic tree was constructed with MEGA 6.0.6 programme by using the neighbor-joining method [17]. Bootstrap analysis (1000 replicates) was performed (Fig. 1).

2.4 Antibacterial Activity Screening of Isolates

To determine the antibacterial activities, isolates were grown in the appropriate medium for 48 hours at appropriate temperatures. Liquid cultures were centrifuged at 8 000 x g for 10 min (4°C). The supernatants of the culture were filtered by using steril milipore filters (0.45 µm pored size, Sartorius). Cell-free supernatants were tested against *Bacillus cereus* ATCC 11778, *Serratia marcescens*, *Pectobacterium carotovorum* DSM 30168, *Enterococcus faecalis* ATCC 51299, *Listeria monocytogenes*, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *Escherichia coli* ATCC 35218, and *Listeria innocua* DSM 20649 as described in Riosen, 2005 [18]. 24 hours after the incubation had started; plates were controlled for inhibition zones.

2.5 Culture-independent Identification of Prokaryotes

2.5.1 DNA isolation from environmental samples

Total DNA from the water sample was isolated by Soil DNA isolation kit according to manufacturers instructions while DNAs were extracted from soil or mud samples according to method decsribed by [19]. Briefly, method is as follows; 0.12 M sodium phosphate buffer (pH 8.0) was added to soil or mud sample (1/1, vol/vol), vortexed and then incubated at room temperature for 10 min before being centrifuged at 7,700 g for 10 min. Then, supernatant was added and a 5 ml PB buffer was added to the pellet and the procedure was repeated again. Lysis solution I (0.15 M NaCl, 0.1 M EDTA, pH 8.0, 10 mg lysozyme ml-1), was used for resuspension of pellet and themix incubated at 37°C for 1 h with occasional gentle mixing. Lysis solution II (0.1 M NaCl, 0.5 M Tris–HCl, pH 8.0, 12% SDS) was added to the mix and suspension freezed at -40°C for 20 min and thawed at 65°C for 20 min and then centrifuged at 7,700 x g for 10 min. This step was repeated once. The supernatant was collected and mixed with 2.7 ml 5 M NaCl and 2.1 ml 10% TRITON-X100 in 0.7 M NaCl and incubated for 10 min at 65°C. CHCl3:isoamyl alcohol (24:1) was added to the mix at equal volume, mixed and centrifuged for 5 min at 3,000 x g. After the supernatant was transferred to a clean tube 13% PEG (polyethylene glycol [8,000 MW] dissolved in 1.6 M NaCl) was added and then the mix was incubated on ice for 30 min and centrifuged at 12,000 x g and 4°C for 10 min. The supernatant was decanted and the pellet was washed with 5 ml 70% cold ethanol and air-dried. Five hundred microliters of deionized H2O was added to the pellet, then the pellet resuspended and two volumes of ethanol were added. After centrifugation at 13,000 x g and 4°C for 30 min. the pellet was washed with 500 70% cold ethanol and air-dried. Finally 500 µL bidistilled water added to DNA extract.

2.5.2 16S *rDNA* gene amplification

To amplify the 16S *rDNA* gene from environmental samples, the isolated total DNAs mentioned above were used. PCR was carried out to amplify 16S *rDNA* gene by using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for bacterial domain. 16S *rDNA* genes were also amplified by using DNA extract from water as a template with archeal F1 (5'-ATTCCGGTTGATCCTGC-3') and 1492R (5'-ACGGHTACCTTGTTACGACTT-3') [20] primers. PCR reaction mix and conditions were the same for archeal and bacterial amplifications as described above.

2.5.3 Clone libraries, sequencing and phylogenetic trees

Clone libraries of environmental samples were constructed using Thermo İnsTAclone PCR kit as described in manufacturer's instructions. *E.coli* DH10B was used as competant cells and 2.5 µL ligation mix was used for bacterial transformation. The clones were grown in AXI

(Ampicillin-X-Gal-IPTG) medium plates which were incubated at 37°C overnight. White colonies were randomly selected for colony PCR in which M13 primers (M13-F:GTAAAACGACGGCCAGT, M13-R:GAGCGGATAACAATTTCACACAGG) were used to amplify 16S *rDNA* fragments. PCR conditions were the same as described previously for archeal and bacterial amplicons. Amplicons were sequenced by ABİ 3730 XL sequencer at MedSanTek (Turkey) and then sequences were compared with those in GenBank using Basic Local Alignment Search Tool (BLAST) to determine the similarities. Reference sequences were chosen based on BLAST similarities and they were used for contruction of phylogenetic trees with those sequences obtained in our study. Sequences were aligned and phylogenetic trees were constructed with MEGA 6.0.6 programme by using the neighbor-joining method [17]. Bootstrap analysis (1000 replicates) was performed (Figs. 2,3 and 4).

3. RESULTS AND DISCUSSION

Ninety-four bacteria which were identified by 16S rDNA sequencing were isolated from six different culture medium and different temperatures (Table 1). As a result of molecular identification, isolates were found to belong to *Bacillus* (n=21), *Halomonas* (n=4), *Enterococcus* (n=1), *Exiquobacterium* (n=2), *Piscibacillus* (n=2), *Haloalkalibacillus* (n=2), *Aerococcus* (n=1), *Acinetobacter* (n=4), *Lysinibacillus* (n=3), *Oceanobacillus* (n=4), *Planococcus* (n=1), *Micrococcus* (n=4) genera and were represented by three different taxa; Firmicutes (n=37), Proteobacteria (n=8), Actinobacteria (n=4). Fourty of the isolates were Gram positive. 83.7% of the ninety-four bacteria were isolated from the medium A, B or C. We assume that high salinity and\or pH values may be the reason for the low isolation yield from the D, E or F medium. However, *Halalkalibacillus, Halomonas* and *Piscibacillus* species were isolated from D, E or F medium. *Halalkalibacillus halophilus* has been previously reported to grow optimally in the presence of 10–15%, (w/v) NaCl, and it has an optimum growth at pH 8.5-9.0 [21]. Optimum NaCl concentration and pH values for genus *Piscibacillus* is %10 and 7.5, respectively [22]. *Halomonas* species have an optimum pH value of 9.5 and they can grow in the presence of 1.5 M NaCl and can be isolated from alkaline-soda lakes [23].

In a study conducted by Deskmukh et al. [7] in Lonar soda lake-India, 74 bacteria were isolated and 11 of them were examined in detail. Microbiological and molecular tests have shown that the isolates belong to the genera *Bacillus, Oceanabacillus, Alcanivorax, Planococcus, Haloalkaliphilum.* Haloalkaliphilic bacteria were also isolated from Sambhar saline lake-India. Ninety-three isolates were divided into 32 groups, and the groups were represented by three different taxa; Firmicutes, Proteobacteria and Actinobacteria [14]. Using similar techniques to ours, members of the *Halobacillus, Bacillus, Exiguobacterium, Oceanobacillus, Halomonas* genera were identified by 16S *rDNA* sequencing. El Hidri et al. [24] was studied for microbial diversity in saline and alkaline environments in Tunisia by using culture-dependent methods. One hundred-twenty two bacteria which were grown at pH 10 and 10% salinity were identified by 16S *rDNA* sequencing. Among the 13 different genera and 20 species, *Oceanobacillus, Bacillus, Halomonas* and *Piscibacillus* were the most common ones in our study. When microbial diversity of Elmentia soda lake in Kenya was examined, it was found that *Halomonas* and *Bacillus* genera were represented by percentage of 37% and 31%, respectively [25]. Others samples of *Marinospirillum, Idiomarina, Vibrio, Enterococcus, Alkalimonas, Alkalibacterium, Amphibacillus, Marinilactibacillus*, *Nocardiopsis* and *Streptomyces* were also found.

Sorokin et al. investigated the diversity of culturable aerobic haloalkalitolerant bacteria with various enzymatic activities from soda soils in Central Asia, Africa, and North America. One hundred-seventy nine isolates were obtained by using media with various polymers at pH 10. As a result of 16S rRNA gene sequence analysis, they found that the bacterial isolates belonged to two large groups—actinomycetes and endospore-forming bacilli. Similar with our results, *B. pseudofirmus* and *B. horokoshii* were found to be dominant among the alkaliphilic or alkalitolerant bacilli [26].

Antibacterial activity screening experiments were achieved using well-diffusion method. Among the tested indicator bacteria, only *M. luteus* was weakly inhibited by culture supernatants of the isolates GBA17, GBA34 and GBA36. There is only a few reports in the literature regarding antibiotic production in alkaline conditions. The low antibiotic production in those conditions may be due to the instability of antibiotics at high pH [1]. *Paecilomyces lilacinus* 1907 [27],

Nocardiopsis dassonvillei OPC-15 [28], *Streptomyces* sp. AK [29], *Streptomyces sannanensis* strain RJT-1 [30], *Streptomyces tanashiensis* strain A2D [31], *B. halodurans* C-125 [32] were reported as antibiotic producers among the alkaliphilic prokaryotes.

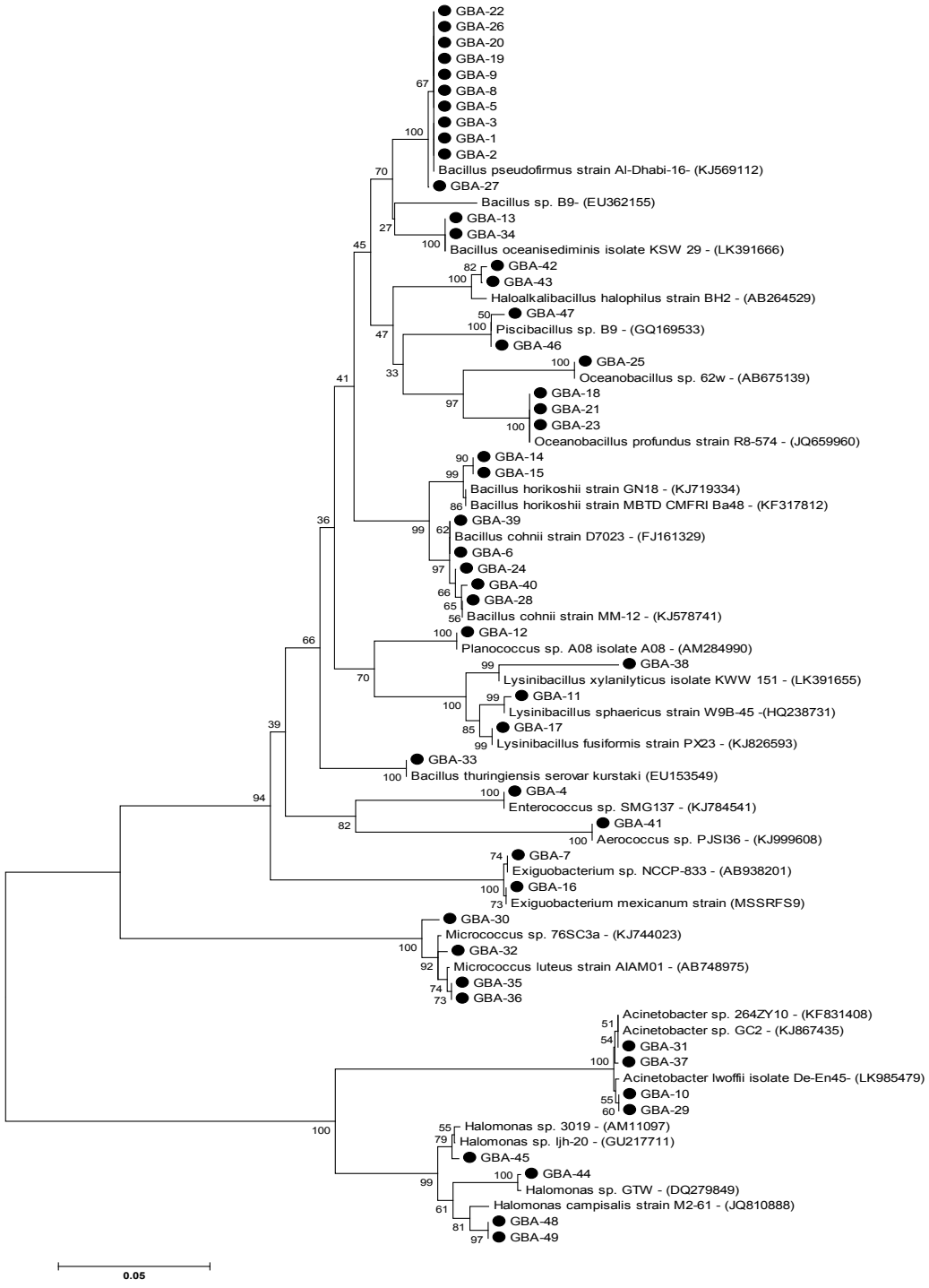

Fig. 1. Phylogenetic positions of alkaliphilic isolates among most related species. Neighbor joining method was used for construction of phylogenetic tree

Table 1. Isolation medium, temperatures and 16S *rRNA* sequence similarities of bacterial isolates

İsolate	Media	Temperature (°C)	Highest similarity-Accession number, Optimum pH values or pH ranges	Similarity rate	Class
GBA-1	A	28	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-2	A	28	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	99%	Bacilli
GBA-3	A	28	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-4	A	28	*Enterococcus* sp. SMG137 - KJ784541,pH=4.5-10 [34]	100%	Bacilli
GBA-5	A	28	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	99%	Bacilli
GBA-6	B	28	*Bacillus cohnii* strain D7023 - FJ161329, pH=9-10 (optimum) [35]	100%	Bacilli
GBA-7	B	28	*Exiguobacterium* sp. NCCP-833 - AB938201, pH=7 [36]	100	Bacilli
GBA-8	B	28	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	99%	Bacilli
GBA-9	B	28	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-10	C	28	*Acinetobacter lwoffii* isolate De-En45- LK985479, ----	100%	Gammaproteobacteria
GBA-11	C	28	*Lysinibacillus sphaericus* strain W9B-45 -HQ238731, pH=7-8 (optimum) [37]	99%	Bacilli
GBA-12	C	28	*Planococcus* sp. A08 isolate A08 - AM284990	99%	Bacilli
GBA-13	C	28	*Bacillus oceanisediminis* isolate KSW 29 - LK391666, pH=7 [38]	100%	Bacilli
GBA-14	C	28	*Bacillus horikoshii* strain GN18 - KJ719334, pH=8 [33]	99%	Bacilli
GBA-15	C	28	*Bacillus horikoshii* strain MBTD_CMFRI_Ba48 - KF317812, pH=8 [33]	99%	Bacilli
GBA-16	C	28	*Exiguobacterium mexicanum* strain MSSRFS9 - KF471138,	100%	Bacilli
GBA-17	C	28	*Lysinibacillus fusiformis* strain PX23 - KJ826593, pH=7-8 (optimum) [37]	100%	Bacilli
GBA-18	A	37	*Oceanobacillus profundus* strain R8-574 - JQ659960, pH=7.5-8.5 (optimum) [39]	100%	Bacilli
GBA-19	A	37	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-20	A	37	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-21	A	37	*Oceanobacillus profundus* strain R8-574 - JQ659960, pH=7.5-8.5 (optimum) [39]	100%	Bacilli
GBA-22	A	37	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-23	A	37	*Oceanobacillus profundus* strain R8-574 - JQ659960, pH=7.5-8.5 (optimum) [39]	100%	Bacilli
GBA-24	B	37	*Bacillus cohnii* strain D7023 - FJ161329, pH=9-10 (optimum) [35]	100%	Bacilli
GBA-25	B	37	*Oceanobacillus* sp. 62w - AB675139, pH=7.5-8.5 (optimum) [37]	100%	Bacilli
GBA-26	B	37	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	100%	Bacilli
GBA-27	B	37	*Bacillus pseudofirmus* strain Al-Dhabi-16- KJ569112, pH=9 [33]	99%	Bacilli
GBA-28	B	37	*Bacillus cohnii* strain MM-12 - KJ578741, pH=9-10 (optimum) [35]	99%	Bacilli
GBA-29	C	37	*Acinetobacter lwoffii* isolate De-En45 - LK985479	100%	Gammaproteobacteria

İsolate	Media	Temperature (°C)	Highest similarity-Accession number, Optimum pH values or pH ranges	Similarity rate	Class
GBA-30	C	37	*Micrococcus* sp. 76SC3a - KJ744023, pH=up to 10 [40]	100%	Actinobacteria
GBA-31	C	37	*Acinetobacter* sp. GC2 - KJ867435	100%	Gammaproteobacteria
GBA-32	C	37	*Micrococcus s*p. 76SC3a - KJ744023, pH=up to 10 [40]	100%	Actinobacteria
GBA-33	C	37	*B. thuringiensis* serovar kurstaki str. YBT-1520- NZ_CP007607	100%	Bacilli
GBA-34	C	37	*Bacillus* sp. B9- EU362155	100%	Bacilli
GBA-35	C	37	*Micrococcus luteus* strain: AIAM01 - AB748975, pH=up to 10 [40]	99%	Actinobacteria
GBA-36	C	37	*Micrococcus luteus*, strain: AIAM01 - AB748975, pH=up to 10 [40]	100%	Actinobacteria
GBA-37	C	37	*Acinetobacter* sp. 264ZY10 - KF831408	100%	Gammaproteobacteria
GBA-38	C	37	*Lysinibacillus xylanilyticus* isolate KWW 151 - LK391655, pH=5-9 [41]	97%	Bacilli
GBA-39	C	37	*Bacillus cohnii* strain D7023 - FJ161329, pH=9-10 (optimum) [35]	99%	Bacilli
GBA-40	C	37	*Bacillus cohnii* strain MM-12 - KJ578741, pH=9-10 (optimum) [35]	99%	Bacilli
GBA-41	B	28	*Aerococcus* sp. PJSI36 - KJ999608,	100%	Bacilli
GBA-42	E	28	*Haloalkalibacillus halophilus* strain BH2 - AB264529, pH=8.5-9 (optimum) [21]	98%	Bacilli
GBA-43	E	28	*Haloalkalibacillus halophilus* strain BH2 - AB264529, pH=8.5-9 (optimum) [21]	98%	Bacilli
GBA-44	F	28	*Halomonas* sp. GTW - DQ279849, pH=9.5 [22]	100%	Gammaproteobacteria
GBA-45	F	28	*Halomonas* sp. ljh-20 - GU217711, pH=9.5 [22]	99%	Gammaproteobacteria
GBA-46	D	37	*Piscibacillus* sp. B9 - GQ169533, pH=7.5 [23]	100%	Bacilli
GBA-47	D	37	*Piscibacillus* sp. B9 - GQ169533, pH=7.5 [23]	99%	Bacilli
GBA-48	F	37	*Halomonas* sp. 3019 - AM11097, pH=9.5 [22]	98%	Gammaproteobacteria
GBA-49	F	37	*Halomonas campisalis* strain M2-61 - JQ810888, pH=9.5 [22]	98%	Gammaproteobacteria

Table 2. Bacterial clones, sequence similarities and divisions

Clone	Source	Highest similarity-Accession number	Similarity rate (%)	Division
GBAM-1	Mud	Bacterium YC-LK-LKJ36 - KP174632	97	Unclassified
GBAM-2	Mud	Bacterium YC-ZSS-LKJ177 - KP174507	90	Unclassified
GBAM-3	Mud	Bacterium YC-ZSS-LKJ62 - KP174611	90	Unclassified
GBAM-4	Mud	Gemmatimonadetes bacterium SCGC AAA240-K19 - HQ675613	88	Gemmatimonadetes
GBAM-5	Mud	*Oceanicella* sp. HME9670 - KF679401	93	Alphaproteobacteria
GBAM-6	Mud	Bacterium YC-LK-LKJ35 - KP174622	99	Unclassified
GBAM-7	Mud	Bacterium YC-ZSS-LKJ90 - KP174612	93	Unclassified
GBAM-8	Mud	Unclassified bacterium clone W4A-A03 -	94	Unclassified
GBAM-9	Mud	*Wenzhouxiangella* sp. XDB06 - KU645983	93	Gammaproteobacteria
GBAM-10	Mud	Bacterium YC-ZSS-LKJ68 - KP174587	93	Unclassified
GBAM-11	Mud	Rhizobiales bacterium NRL2 - CP016093	89	Alphaproteobacteria
GBAM-12	Mud	Bacterium YC-ZSS-LKJ62 - KP174611	94	Unclassified
GBAM-13	Mud	Bacterium YC-ZSS-LKJ193 - KP174603	99	Unclassified
GBAM-14	Mud	*Luteolibacter yonseiensis* strain EBTL01 - JQ319003	97	Verrucomicrobia
GBAM-15	Mud	Bacterium YC-LK-LKJ3 - KP174636	87	Unclassified
GBAM-16	Mud	Bacterium YC-ZSS-LKJ107 - KP174511	94	Unclassified
GBAM-17	Mud	Unclassified eubacterium from the Amazon - U68600	92	Unclassified
GBAW-18	Water	Bacterium YC-ZSS-LKJ111 - KP174593	90	Unclassified
GBAW-19	Water	*Puniceicoccus vermicola* strain IMCC1545 - DQ53904	95	Verrucomicrobia
GBAW-20	Water	Bacterium YC-ZSS-LKJ53 - KP174548	97	Unclassified
GBAW-21	Water	Bacterium WH3-1 - JQ269304	88	Unclassified
GBAW-22	Water	Bacterium YC-ZSS-LKJ53 - KP174548	97	Unclassified
GBAW-23	Water	*Lewinella agarilytica* strain SST-19 - AM286229	88	Bacteriodetes
GBAW-24	Water	Unclassified bacterium clone K2-S-20 -AY344428	96	Unclassified
GBAW-25	Water	*Thalassococcus* sp. MME-075 - KP410684	91	Alphaproteobacteria
GBAW-26	Water	*Puniceicoccus vermicola* strain IMCC1545 - DQ539046	93	Opitutae
GBAW-27	Water	Hyphomonadaceae bacterium Bac120R - KP795813	99	Alphaproteobacteria
GBAS-28	Soil	*Chryseobacterium* sp. MYb151 - KX079857	99	Bacteriodetes

Table 3. Archeal clones, sequence similarities and divisions

Clone	Highest similarity-Accession number	Similarity rate (%)	Division
GAAW-1	*Halorhabdus* sp. PJ16 - KJ644204	93	Halobacteria
GAAW-2	*Salinirubrum litoreum* strain XD46 - JQ237118	92	Halobacteria
GAAW-3	*Natronomonas* sp. YPL13 - KT261635	96	Halobacteria
GAAW-4	Halobacteriaceae archaeon GSLN9 - KR012970	86	Halobacteria
GAAW-5	*Halomarina oriensis* strain KeC-11 - NR_112987	89	Halobacteria
GAAW-6	Archaeon PY-9 - JQ268003	80	Unclassified
GAAW-7	Bacterium YC-ZSS-LKJ135 - KP174509	97	Unclassified
GAAW-8	*Halogranum amylolyticum* strain JCM 16428 - NR_113451	99	Halobacteria
GAAW-9	*Halorubrum* sp. Ea4p - HG421005	99	Halobacteria
GAAW-10	Halobacteriaceae archaeon SY-39 - KM656492	94	Halobacteria
GAAW-11	*Halorhabdus* sp. PJ16 - KJ644204	92	Halobacteria
GAAW-12	*Halomicrobium* sp. MHC-15-3 - AB850661	97	Halobacteria
GAAW-13	Haloarchaeon HTSR1 - NZ_CP016070	93	Halobacteria
GAAW-14	*Halocalculus aciditolerans* - AB844670	95	Halobacteria

The sequence analysis results were obtained from TA cloning of PCR amplified 16S rDNA from environmental samples which was followed by the selection of white colonies, PCR amplification with M13 primers were reported in this paper. BLAST analysis results of sequences results with lengths between 338-761 bp for Bacteria domain from mud (n=17), water (n=10) and soil (n=1) samples are given in Table 2. Fourteen sequences were obtained from water sample for archea according to the PCR amplified 16S rDNA genes. The BLAST results for archea were given in Table 3.

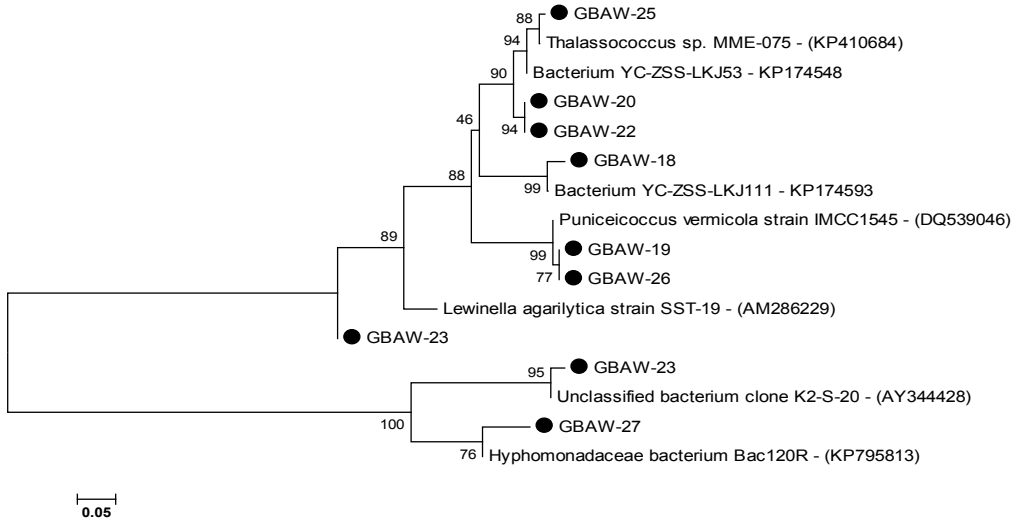

Fig. 2. Phylogenetic positions of bacterial clones from water sample among most related species. Phylogenetic positions of alkaliphilic isolates among most related species

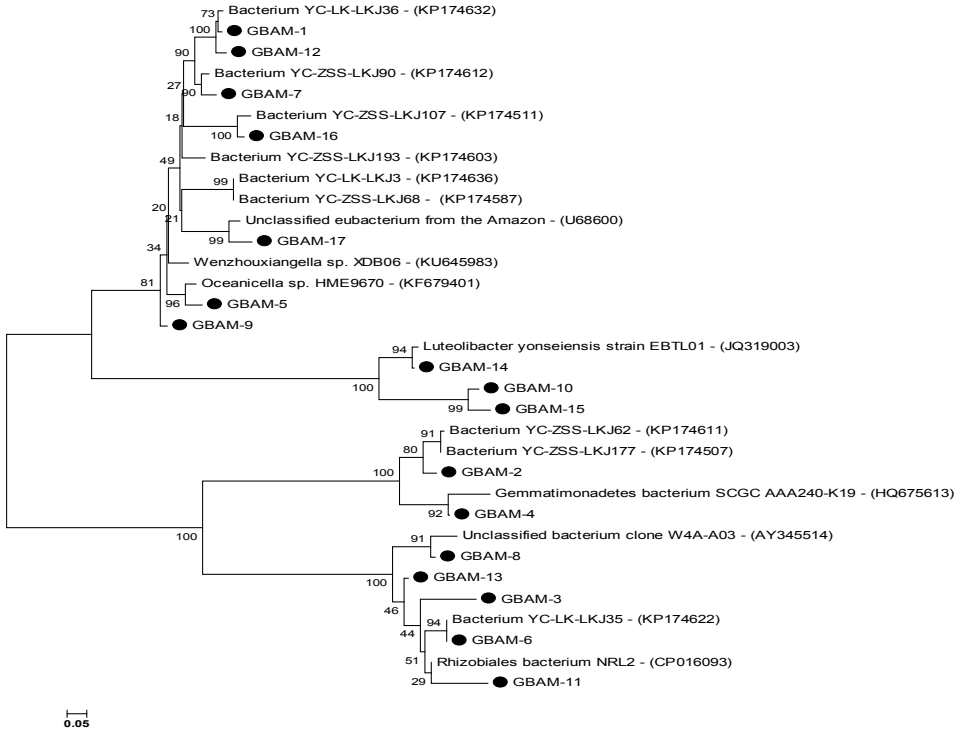

Fig. 3. Phylogenetic positions of bacterial clones from mud sample among most related species. Phylogenetic positions of alkaliphilic isolates among most related species

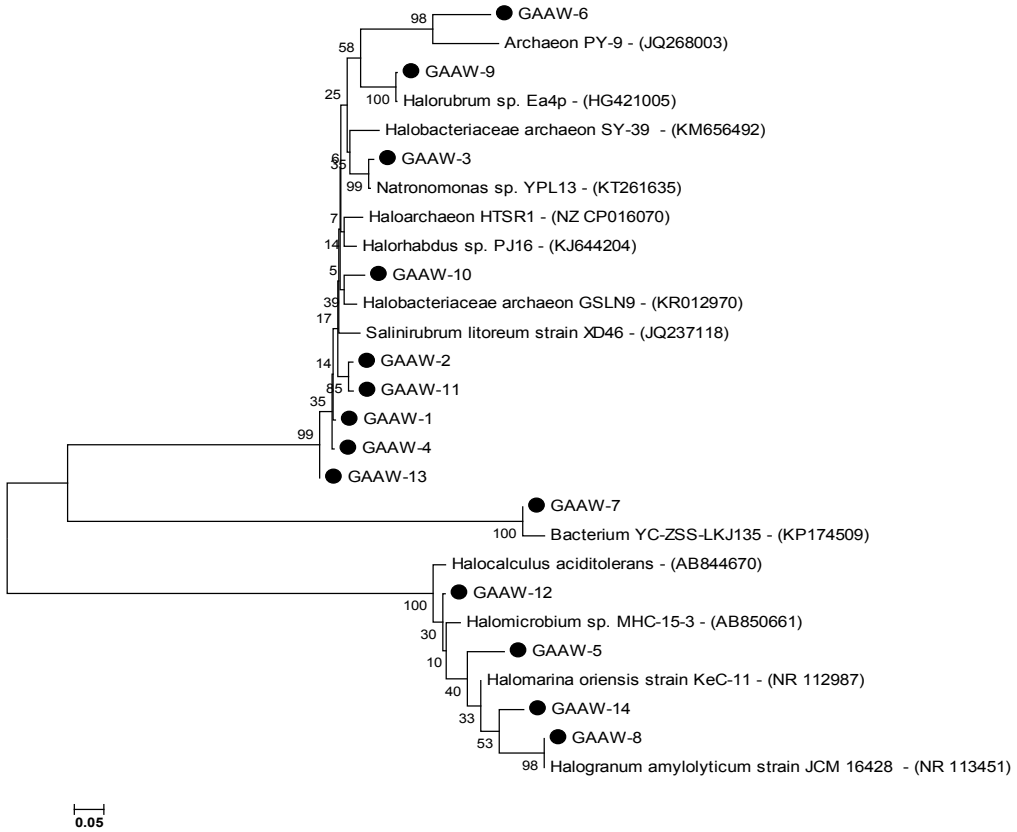

Fig. 4. Phylogenetic positions of archeal clones from water sample among most related species. Phylogenetic positions of alkaliphilic isolates among most related species

According to our BLAST analysis, a majority of the clones (45%) showed highest similarities with unclassified members of the domain Bacteria, especially with Bacterium YC which were obtained from Yuncheng salt lake, China. The rest of the bacterial diversity was found to be classified as Gemmatimonadetes (n=1), alphaproteobacteria (n=4), Flavobacteria (n=1), Opitutae (n=2), Gammaproteobacteria (n=1), Saprospira (n=1), Verrucomicrobia (n=1). All of the identified archeal clones belong to the orders of Class Halobacteria; 12 are members of order Halobacteriales and two are order Haloferaceles. Sequence similarity for bacterial clones was between 87-99% and 80-99% for archeal groups.

When the 16S rDNA sequence of bacterial isolates were compared to sequences of clones obtained directly from environmental samples, there were no common species or genera found. This is most likely because the culture medium used for the incubation does not support the growth of extreme halophilic and alkaliphilic bacteria/archea.

In a work conducted on soda lakes in Russia, microbial diversity was revealed by culture-independent approach [42]. Result of 16S rDNA sequencing showed that the most abundant taxa present in temperate salinity were Bacteroidetes, Alpha- and Gamma-proteobacteria, while the members of Euryarcheota were dominant at high salinity. Similar to our results, Aguirre-Garrido et al. [8] also found the halophilic and halotolerant microorganisms were the most dominant bacterial communities in soda crater lakes. Wani et al. [43] searched for microbial diversity in Lonar Soda Lake in India by molecular methods and they constructed a 16S rDNA library with 500 clones affiliated to genera *Bacillus, Alkaliphilus, Pseudomonas*, *Halomonas.* Simachew et al., investigated the effect of the salinity on prokaryotic diversity in Abijata-Shalla Soda Ash Concentration Pond system situated in Ethiopian Rift Valley. As a consequence of 16S rRNA pyrosequencing, dominant prokaryotic phyla were found to be consisted of *Planctomycetes, Bacteroidetes, candidate division TM7, Deinococcus-Thermus, Firmicutes,*

Actinobacteria, Proteobacteria, and *Euryarchaeota.* Researchers also emphasized that prokaryotic diversity richness decreased with increasing salinity in the pond [44].

Lake Van is an another example of an extremely high alkaline environment which is located in Eastern Anatolia, Turkey. Lopez-Garcia et al. [45] studied the molecular diversity of Lake Van based on 16S rDNA amplification. Members of the bacterial classes Alpha-, Beta- and Gammaproteobacteria, the Cyanobacteria, the Cytophaga-Flexibacter-Bacteroides (CFB) group, the Actinobacteria and the Firmicutes were observed in the surface and internal microbialite samples. Budakoglu et al. [46] studied the archeal microbial diversity in Lake Acıgöl, using culture-independent methods and six clones were described in his study. According to 16S rDNA sequence analysis Budakoglu has suggested that phylotypes were affiliated with the genera *Halorubrum, Halosimplex, Halorhabdus*, *Haloterrigena* and *Natronococcus.* In another study, prokaryotic diversity of three alkaline lakes; Lake Acigol, Lake Salda and Lake Yarisli were investigated by Next Generation Sequencing [47]. The examples of bacterial classes found in Lake Acıgöl are as follows; Alphaproteobacteria, Cyanobacteria, Bacilli, Gammaproteobacteria, and Actinobacteria. The researchers have also reported that Methanobacteria, Halobacteria, Thaumar-chaeota, Thermoplasmata and Methanomicrobia are the dominant archeal classes. The difference in our study from those mentioned above is that we have used both microbiological and molecular methods to determine the prokaryotic diversity. We have also investigated the antibacterial properties of the isolated strains.

Alkaliphilic or haloalkaliphilic bacteria, especially members of genus *Bacillus* are very important for bioprospecting studies due to their capacity for high production of enzymes, organic acids, optical pigments, exopolysaccharides, etc [48]. Our next aim is to search for enzymes and the other biotechnologically usefull products from extremophilic isolates obtained in this study.

4. CONCLUSION

1. In this study, we have obtained a high yield of bacterial isolates when using A, B or C medium for the isolation of the chemoorganotrophic alkaliphilic bacteria compared to the strains obtained from D, E or F medium.
2. When the 16S rDNA sequence of bacterial isolates were compared to sequences of clones obtained directly from environmental samples, there were no common species or genera found.
3. The bacterial strains isolated in this study have properties such as salinity and alkalinity tolerance. Therefore, they are ideal candidates for biotechnologically important products such as enzymes, antibiotics and carotenoids.

ACKNOWLEDGEMENT

This work was supported by Adnan Menderes University, Scientific Research Projects (ADÜ BAP-13009).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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