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Microbial Communities Inhabiting Hot Springs Water of Jazan Area, Saudi Arabia

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Hot springs water is a natural habitat for thousands of microbial species;there are approximately ten hot springs scattered throughout Saudi Arabia; however, the microorganisms in these springs have not been thoroughly investigated and characterized. In this study, water samples from two hot springs of Al-Khoba and Al-Arida in Jazan area located at the Southern part of Saudi Arabia were collected and evaluated. Specifically, microbial communities were analyzed by the amplification of 16S rRNA gene sequences, followed by DNA sequencing and phylogenetic analysis. *Tepidimonas taiwanensi* was the dominant species in both samples with 49% and60%, while species closely related to Uncultured Bacteroidetes bacterium EW2-030 and *Paenibacillusalginolyticus* comprised the second largest groups present in samples with 35% and19% from Al-Khoba and Al-Arida, respectively.

Key words: Hot springs water, Jazan, Clone library, Phylogenetic analysis.

Extremophiles are microbes that have the ability to survive and maintain their life cycle in severe environments such as those characterized by low/high pH (acidophiles/alkaliphiles), high salt (halophiles), high pressure (piezophiles), low temperature (psychrophiles) and high temperature (hyperthermophiles)[29]. Many samples collected from extreme conditions have been shown to have the potential for use in biotechnological and industrial applications[29,28] owing to adaptation to their native habitats. Microorganisms capable of growth at 50–75°C have been intensively investigated[30], and application of heat stress to microbial communities of hot springs[1] has revealed that *Bacilluslicheniformis*, *Bacillus pumilus*, *Synechococcus lividus* and *Chloroflexus aurantiacus* have the potential to tolerate such conditions. Moreover, new biotechniques are still contributing in the isolation and characterization of novel microorganisms. Many of these techniques involve combining culture-dependent and independent techniques to fully characterize the indigenous microbial diversity in such ecological systems[31].

The automated ribotyping system (RiboPrinter®) was employed in conjunction with BIOLOG and VITEK to generate a microbial profile from hot springs water sampled from Eski_ehir, Turkey[21]. Phenotypic characteristics based on traditional methods and genotypic characteristics based on 16S rRNA gene sequence analysis of collected samples from Soldhar in the Indian Himalayas showed that the microbial community mainly consisted of *Geobacillus stereo thermophilus* (11 strains), as well as *Geobacillus kaustophilus* and *Geobacillus sp*[23]. Similarly, samples collected from five different hot springs

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in Jordan mainly contained *Geobacillus stearo thermophilus* (5 strains), while only one spring contained *Geobacillusthermoglucosidasius*[18]. Profiling of microbial communities in water from the acidic hot springs of Kamojang geothermal field in West Java, Indonesia using both culture-dependent and independent methods revealed the presence of Chrenorchaeota and Proteobacteria, while Firmicute and gamma Proteobacteria were identified by culture-dependent techniques[2,4]. An advanced approach and mechanism supported by sequencing of cloned PCR products and quantitative PCR (qPCR) of 16S rRNA and metabolic genes led to identification of three Aquificaegenera related to *Thermocrinis*, *Hydrogenobaculum* and *Sulfurihydrogenibium* and other members belonging to Proteobacteria (Alpha, Beta andGamma), Firmicutes, Acidobacteria and Deinococcus-Thermus[11]. To isolate thermo-tolerant bacteria from several hot springs water samples collected from Siwa, Matrouh, Egypt, around 300 bacterial cultures were isolated and exposed to thermal stress at 65°C. RAPD, Box-PCR and 16S rRNA sequence analysis revealed that *Bacillus licheniformis*and *Bacillus pumilus* responded to the stress[1]. Additionally, a study conducted by a research group in Taiwan coupled several classical methods and advanced biotechnological techniques to identify a novel alkaline-protease-producing bacterium, *Tepidimonas taiwanensis* sp. nov., isolated from a hot spring around Pingtung in southern Taiwan[8].

Despite of the majority of Saudi Arabia being arid and subject to extreme weather, there are about ten hot spring systems in the country[19]. These springs have not yet been fully investigated, although a few studies were carried out to characterize the microbial communities and geochemistry of geothermal springs around Jazan[13,12,63]. Most of the active hot springs in Saudi Arabia are located in the south, which is characterized by rocky hills and mountains. The two most famous hot springs in the city of Jazan are Al-Khoba and Al-Arida. These springs have high levels of sodium and potassium (around 335 ppm) and low levels of nitrate[6]. A recent study revealed the presence of six species of bacteria among 15 isolates in several samples collected from springs in Jazan: *Bacillus cereus, B. licheniformis, B. thermoamylovorans, Pseudomonas* sp., *Pseudomonas aeruginosa*and *Enterobacter sp*[13]. Moreover, three strains of thermophilic bacteria isolated from Al-Khoba and Al-Arida were found to be highly homologous with *Bacillus* sp., *Brevibacillusborstelenesis*and*Deinococusgeothermals*[12]. The present study was conducted to investigate the microbial communities of Al-Khoba and Al-Arida hot springs water in Jazan, Saudi Arabia in detail.

MATERIALS AND METHODS

Hot springs water sampling

Two hot springs water samples were collected from Jazanlocated in southern part of Saudi Arabia at longitude 42°–43°E and latitude 16°–17°N. Al-Khoba is located approximately 53 km southeast of the city of Jazan, while Al-Arida is located 33 km northeast of the city.The samples were collected in 1000 ml sterilized polypropylene bottles; Temperature and PH of water samples has been taken immediately. Temperature ranged between 56 to 60 °C and PH of 7.5 to 8.5 in both samples, after that samples werekept on ice until transfer to the laboratory, after which they were stored at 4°C until analysis.

DNA Extraction and Amplification of 16S rRNA Gene Sequences

DNA was extracted from water samples using a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, CA, USA). Hot springs water samples were extracted in triplicate and pooled due to low biomass. An approximately 1490 bp fragment of the 16S rRNA gene was amplified from the samples using the broad-specificity primers 8F[10] and 1492R[14]. PCR reactions were performed using a BioRadiCycler (BioRad, Hemel Hempstead, UK). Takara Ex Taq Polymerase (Millipore UK Ltd., Watford, UK) was used to amplify DNA from the sample extract. The PCR amplification protocol used with the 8F and 1492R primers was as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of melting at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 3 min. and a final extension step at 72°C for 5 min. The purity of the amplified products was determined by electrophoresis in Tris-acetate-EDTA (TAE) gel followed by staining of DNA with ethidium bromide and observation under short wave UV light using a BioRadGeldoc 2000 system

(BioRad, Hemel Hempstead, Herts, UK).

Cloning

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK) and ligated directly into a cloning vector containing topoisomerase I-charged vector arms (Agilent Technologies, Wokingham, UK) prior to transformation into *E. coli* competent cells expressing Crerecombinase (Agilent Technologies, Wokingham, UK). White transformants that grew on LB agar containing ampicillin and X-Gal were screened for inserts by PCR using primers complementary to the flanking regions of the PCR insertion site of the cloning vector. The PCR method for screening consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of melting at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The resulting PCR products were purified using an ExoSap protocol. Briefly, 2 µl of ExoSap mix (0.058µl Exonuclease I, 0.5µl Shrimp Alkaline Phosphatase and 1.442 µl QH_2O) were added to 5 µl of PCR product and the samples were then incubated at 37°C for 30 min followed by 80°C for 15 min.

DNA Sequencing

Nucleotide sequences were determined by the dideoxynucleotide method using an ABI Prism BigDye Terminator Cycle Sequencing Kit in combination with an ABI Prism 877 Integrated Thermal Cycler and an ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Warrington, UK). Sequences (typically 900 base pairs in length) were analysed using Mallard[5] to check for the presence of chimeras or sequencing anomalies. Operational taxonomic units (OTU) were determined at a 98% sequence similarity level using Mothur[22].

Phylogenetic Analysis

The individual OTU sequences were analysed using the sequencing database of known 16S rRNA gene sequences provided by the Ribosomal Database Project[9] to identify the nearest neighbours. The sequences obtained were submitted to a BLAST search to retrieve the corresponding phylogenetic relatives. Phylogenetic affiliations were confirmed by analyses of all related species recognized by the taxonomic and classification hierarchy using the NCBI Taxonomy database. Three neighbour-joining phylogenetic trees were constructed to analyse the relationships among sequences of the ribosomal library and related organisms from the GenBank database. Phylogenetic analysis was conducted using the MEGA5.10 software[26].

RESULTS

Microbial Community Analysis

In this study, the microbial diversity of two hot springs in the Jazan area of southern Saudi Arabia was investigated using a clone library constructed from amplified community 16S rRNA genes. Overall, 145 clones (57 from Al-Khoba and 88 from Al-Arida) were obtained and identified by sequencing. Tables 1and2show the closest matching 16S rRNA sequences for microbial communities present in Al-Khoba and Al-Arida, respectively. Figures 1 and 2 summarize the microbial communities present within the samples. The dominant phylogenetic class in both samples was β-Proteobacteria, which comprised ~51% of the clone library in Al-Khoba and ~64% of the clone library in Al-Arida. Al-Khoba was found to contain β-Proteobacteria most closely related to *Tepidimonas taiwanensis*(T) I1-1, *Oxalobacteraceae bacterium* HTCC315 and the Uncultured Betaproteobacterium CrystalBog021G5. In the Al-Arida sample, the closest matches to the β-Proteobacteria were Uncultured *Hydrogenophilus* sp. CSR-2 and *Tepidimonas taiwanensis* (T) I1-1. The second largest class present in Al-Khoba consisted of members of Bacteroidetes, which accounted for ~42% of the clone library. Among these, the closest matching microorganisms identified were uncultured bacterium B3-3, Uncultured Bacteroidetes bacterium EW2-030, uncultured bacterium c26T103b and uncultured bacterium Tatahouine-EC11. The second largest class of microorganisms within the Al-Arida sample belonged to the family Paenibacillaceae, which comprised ~23% of the clone library. Among these, the closest matching microorganisms were found to be *Cohnellaluojiensis* HY-22R and *Paenibacillus alginolyticus* (T) DSM 5050. In addition, δ-Proteobacteria, Sphingobacteria, Clostridia, δ-Proteobacteria andunclassified bacteria were found in Al-Khoba, whereas Spartobacteria, Clostridia, Deinococci, Nitrospira, δ-Proteobacteria, α-

Table 1. Closest cultured strains of bacteria based on 16S rRNA gene libraries of hot springs water from Al-Khoba

Phylogenetic Class	Closest Match	% Clone Library	% Match
β-proteobacteria		50.6	
	Tepidimonastaiwanensis(T) I1-1	48.4	(99.0)
	Oxalobacteraceae bacterium HTCC315	1.1	(98.1)
	Uncultured β-proteobacterium; CrystalBog021G5	1.1	(96.4)
Bacteroidetes		41.8	
	Uncultured bacterium B3-3	2.2	(99.7)
	Uncultured Bacteroidetes bacterium EW2-030	35.2	(98.7)
	Uncultured bacterium c26T103b	1.1	(95.0)
	Uncultured bacterium Tatahouine-EC11	3.3	(95.5)
Sphingobacteria		3.3	
	*Algoriphagusolei*CC-Hsuan-617	3.3	(98.1)
ε-proteobacteria		1.1	
	Uncultured μ-proteobacterium MilliWH10	1.1	(97.8)
Clostridia		1.1	
	Uncultured bacterium TSNIR003_D08	1.1	(99.7)
-proteobacteria		1.1	
	Uncultured bacterium Q3-6C17	1.1	(95.5)
Unknown		1.1	
	Uncultured bacterium SUL_886_L02	1.1	(95.5)

Table 2. Closest cultured strains of bacteria based on 16S rRNA Gene libraries from hot springs water from Al-Arida

Phylogenetic Class	Closest Match	% Clone Library	% Match
β-proteobacteria		63.6	
	Uncultured *Hydrogenophilus* sp. CSR-2	3.4	(95.4)
	Tepidimonastaiwanensis (T) I1-1	60.2	(98.5)
Bacilli		22.7	
	Cohnellaluojiensis HY-22R	3.4	(98.9)
	Paenibacillusalginolyticus (T) DSM 5050	19.3	(98.1)
α-proteobacteria		3.4	
	Uncultured bacterium; HDBW-WB01	3.4	(98.8)
Deinococci		2.3	
	Meiothermusruber 16106	2.3	(99.0)
Clostridia		1.1	
	Uncultured bacterium xmg-22	1.1	(94.2)
Sphingobacteria		2.2	
	Thermonemarossianum SC-1	1.1	(99.3)
	Uncultured bacterium 654919	1.1	(97.6)
Nitrospira		1.1	
	Uncultured bacterium SsB101	1.1	(94.1)
ω-proteobacteria		1.1	
	Uncultured *Desulfomonile sp.* clone 618	1.1	(96.1)
Spartobacteria		1.1	
	Uncultured Verrucomicrobia bacterium F1bUB08T3	1.1	(96.3)
γ-proteobacteria		1.1	
	Uncultured bacterium TX1A_25	1.1	(95.7)

Proteobacteria, α-Proteobacteria and Sphingobacteria were detected in the Al-Arida sample.

Evolutionary history was inferred from both samples using the Neighbor-Joining method. Both bootstrap consensus trees were inferred from 500 replicates and are taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in

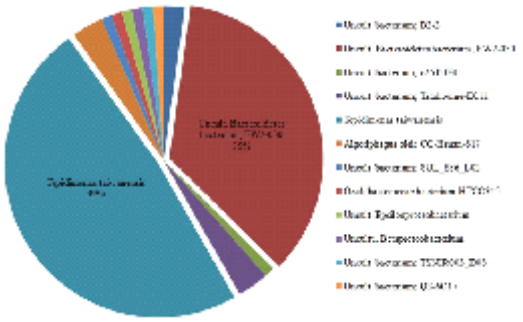

Fig. 1. Closest matching microorganisms at Alkhoba based on 16S rRNA

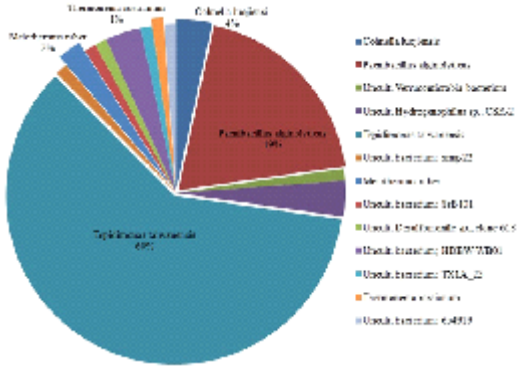

Fig. 2. Closest matching microorganisms at Al-Arida based on 16S rRNA

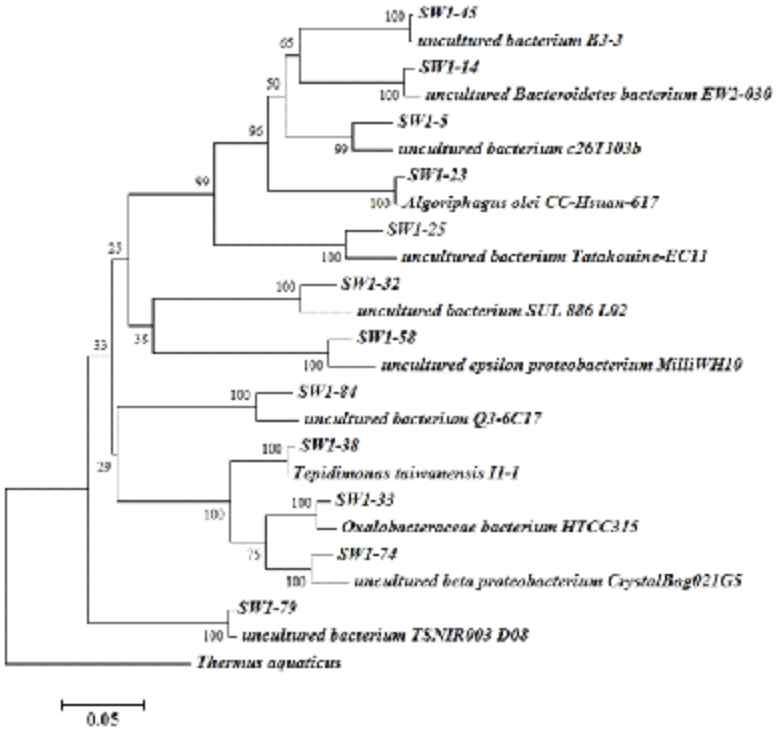

Fig. 3. Bootstrap consensus tree for Al-Khoba inferred from 500 replicates showing the β-Proteobacteria, Bacteroidetes, μ-Proteobacteria, Sphingobacteria, Clostridia, and δ-Proteobacteria 16S rRNA gene phylotypes of bacterial sequences that were detected in the DNA library and closely related sequences identified based on a BLAST search of the NCBI database

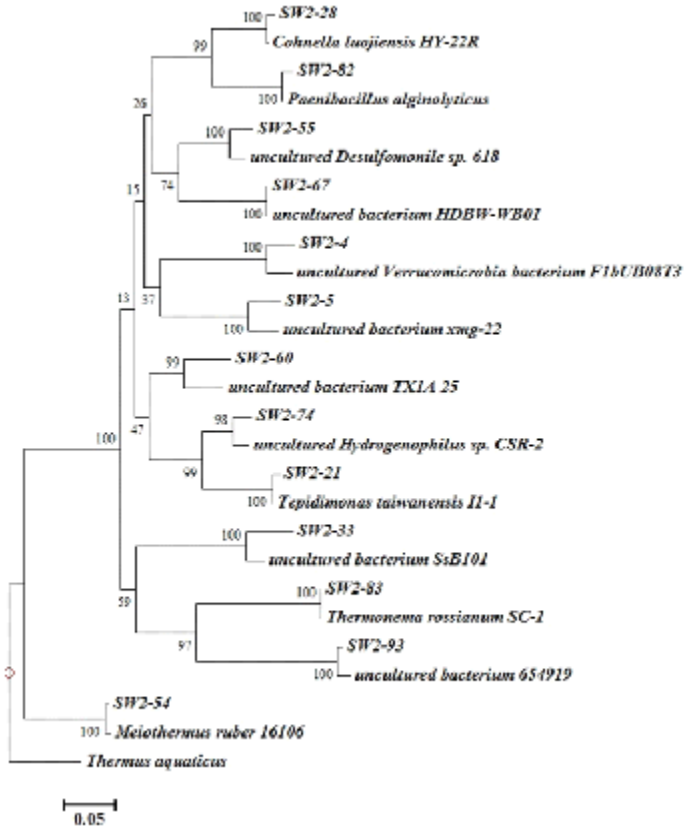

Fig. 4. Bootstrap consensus tree for Al-Arida inferred from 500 replicates showing the β-Proteobacteria, Bacillus,Spartobacteria, Clostridia, Deinococci, Nitrospira, δ-Proteobacteria, α-Proteobacteria, γ-Proteobacteriaand Sphingobacteria,16S rRNA gene phylotypes of bacterial sequences that were detected in the DNA library and closely related sequences identified based on a BLAST search of the NCBI database

the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences (Al-Khoba tree) and 27 nucleotide sequences (Al-Arida tree). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

DISCUSSION

Microorganisms have long been known to play essential roles in natural environments, particularly in extreme environments such as hot springs. There are approximately ten hot springs located throughout Saudi Arabia; however, there have been few investigations of the microbial communities in these systems. A more detailed understanding of bacterial diversity within these hot springs is essential in understanding the implications of their use from various perspectives. Additionally, it is hoped that hot springs water isolates can be utilized for further biotechnological developments and applications. This study attempted to characterize the microbial communities present within hot springs in Jazan, Saudi Arabia.

In this study, microbial characterization was combined with determination of dissolved chemical species in two different samples of hot springs water. Bacterial classes identified included α-Proteobacteria, β-Proteobacteria, γ-

Proteobacteria, ´-Proteobacteria,Bacteroidetes, Bacilli, Clostridia, Deinococci, Nitrospira, Spartobacteriaand Sphingobacteria.The results of this study were similar to those of previous studies[13,12] conducted in Saudi Arabia in the Jazan area, although the present study revealed diverse microbial communities in hot springs water. The dominant bacterial class in both samples was ²-Proteobacteria, while other significant constituents of the 16S rRNA clone libraries included members of Bacteroidetesin the Al-Kobha hot springs and Paenibacillaceae in the Al-Arida hot springs. Thermophilic *Bacillus* spp. have also been isolated from a number of hot springs globally[13,12,23,1] and most species of *Bacillus* can produce important enzymes that are useful in a variety of applications including food production, leather tanning and environmental remediation.

The results of a BLAST search indicated that the dominant species, which represented 48.4% and 60.2% of the total population in the Al-Khoba sample and Al-Arida sample, respectively, was most closely related to *Tepidimonastaiwanensis*(T) I1-1. This organism was first isolated from hot springs located in the Pingtung area of Southern Taiwan[8] and is capable of producing alkaline protease. In addition, the absence of nitrate from both samples confirmed the growth of *Tepidimonas taiwanensis*s pp[8]. The second most abundant group found in hot springs of Al-Khoba were members of the class Bacteroidetes (41.8%), with the closest matches being to Uncultured bacterium B3-3, Uncultured Bacteroidetes bacterium EW2-030, Uncultured bacterium c26T103b and Uncultured bacterium Tatahouine-EC11. Clone EW2-030 accounted for 35.2% of these isolates. This organism has previously been detected in Lake Elmenteita in Kenya[15] and is known to be tolerant to alkaline, saline and high chloride and sulfate conditions[15]. Conversely, the isolates from Al-Arida hot springs most closely matched *Paenibacillus alginolyticus*(T) DSM 5050, *Cohnellaluojiensis* HY-22R, *Meiothermusruber* 16106 and *Thermonemarossianum* SC-1, which comprised 19.3, 3.4, 2.3 and 1.1% of the entire microbial community, respectively. *Paenibacillus alginolyticus* has been fully described and characterised elsewhere[25,16], but almost no information is available regarding strain DSM5050. In general, *Paenibacillus* spp. have very important applications[20] such as in laundry detergents. *Cohnellaluojiensis* was first isolated from the soil of a Euphrates poplar forest in Xinjiang, China[7], while *Meiothermusruber* has been fully described elsewhere[17,24]. *Thermonemarossianum* was first isolated from saline hot springs along the Bay of Naples, Italy[27]. These four species do not reduce nitrate and can survive under high NaCl conditions. Additionally their optimum growth occurs around 65–70C°, which makes them thermophilic bacteria.

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Conflict of Interest

The author declares that he has no conflict of interest.

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