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*Full Length Research Paper*

# Culture-dependent and culture-independent approaches to study the bacterial and archaeal diversity from Jordanian hot springs

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**The microbial diversity was investigated at five major hot springs in Jordan (Ashounah, Waggas, Zara, Zarqa Ma'in and Afra springs) using both microbiological culture-based and molecular culture-independent approaches. One hundred and thirty two (132) bacterial isolates were obtained and characterized morphologically. Out of 132 isolates, 125 isolates were gram positive rods, while the other seven isolates were gram positive coccobacilli. The bacterial growth at different high temperatures was determined, and revealed that 19 out of 132 isolates were able to grow at high temperatures of up to 75°C. All bacterial isolates grew at pH 7.0, except one single isolate from Zarqa Ma'in spring which was able to grow at pH 3.5. Culture-independent approaches using polymerase chain reaction (PCR) was used for the amplification of specific 16S rDNA sequences of *Bacteria, Archaea, Green sulfur bacteria, Green nonsulfur bacteria, Heliobacteria, and* methanogenic *Archaea* from metagenomic DNA extracted directly from water and mat samples from each thermal spring. Nine water samples and 9 mat samples from all spring sites revealed the presence of bacteria yielding the amplicon size of (1500 bp), while 4 water samples from Zarqa Ma'in springs and 5 mat samples from Zarqa Ma'in springs and Waggas well revealed the presence of archaea with an amplicon size of (650 bp). When the primer pair targeting purple phototrophic bacteria was used, positive PCR amplification product (229 bp) appeared in 6 mat samples from Zarqa Ma'in, Afra, Zara, Ashounah and Waggas springs. Also PCR identification of bacterial isolates using primer pair specific to the 16S rDNA gene sequences of the genus *Bacillus* indicated that 96.97 % (128 out of 132) of bacterial isolates have the size of PCR amplicon (320 bp) for the genus *Bacillus*.**

**Key words:** Bacteria, archaea, diversity, hot springs, Jordan.

## INTRODUCTION

The classification scheme of organisms recognizes three major domains of living organisms, archaea, bacteria and eukarya (Allers et al., 2005). Living organisms are usually divided into several classes based on the temperature at which they grow well. In general, moderate thermophiles are primarily bacteria and display optimum growth temperature between 60 and 80°C, hyperthermophiles are primarily archaea and grow optimally at 80°C or above (Andrade et al., 1999). Understanding the adaptations that enable thermophilic organisms to survive at extreme temperature is a challenge that has interested researchers (England et al., 2003; Saraboji et al., 2005; Koskinen et al., 2008). Temperature variation is a special feature because it can penetrate physical barriers and can have dramatic effects on the structure of macromolecules, and also affect all levels of biological adaptation (Hickey et al., 2004).

The most common and accessible thermal habitats for thermophilic microorganisms are hot springs, sulfatara and geothermally heated soils (Marteinsson et al., 2001; Takai et al., 1999). The study of extreme environments has considerable biotechnological potential; an example is the *Taq* DNA polymerase, purified from the hot springs bacterium *Thermus aquaticus* (Chien et al., 1976). The recent interest in biotechnology, coupled with the

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discovery of novel thermophiles, has prompted studies on the utilization of thermophiles and their enzymes for industrial purposes (kanokratana et al., 2004).

Molecular phylogenetic techniques based on 16S rRNA gene sequence comparisons have been employed to investigate microbial diversity in several habitats, terrestrial hot spring microbial communities were among such habitats to be surveyed with this technology (kanokratana et al., 2004; Ward et al., 2002). The application of these techniques to study natural microbiotas in hot springs without the traditional requirement for cultivation has allowed the identification and study of many previously undetected organisms (kanokratana et al., 2004; Hobel et al., 2005; Skirnisdottir et al., 2000), since typically only a small fraction (<1%) of naturally occurring microorganisms is routinely cultivatable by standard techniques (Barns et al., 1996; Handelsman, 2004; Skirnisdottir et al., 2000).

To date, a number of extreme thermophiles or hyperthermophiles within the domain of bacteria and archaea have been isolated from terrestrial hot springs (Ghosh et al., 2003; Marteinsson et al., 2001; Takai et al., 1999; Meyer-Dombard et al., 2005; Skirnisdottir et al., 2000). A great diversity of bacteria and archaea in hot spring samples from Yellowstone National Park (USA) was revealed by analysis of rRNA genes that were amplified by the polymerase chain reaction from environmental DNA (Barns et al., 1996; Burton et al., 2000; Merey-Dombard et al., 2005).

In Jordan, several hot springs distributed in different regions having temperature ranges between 20 and 63°C have been known by geologists for many years, geographical distribution, chemical and physical characteristics of these springs were described (Harahsheh, 2002; Sunna, 2004; Swarieh, 2000).

There are about 200 thermal springs in Jordan distributed across the country. The major sites are distributed in three regions. The north region includes Al Hammah springs with water temperature that is up to 43°C, North shounah well (57°C), Abu Dablah spring (37°C), Al Mansheyyah, Waggas and Abu Ziad wells (51.5°C), Deir Alla (35°C), Mua'ddi springs (28 - 36°C) and Jerash spring (28°C). The middle region includes Zarqa Ma'in springs (47 - 63°C), Zara springs (53 - 54°C), Al Kafrain wells (32.8 - 35.5°C) and Al Azraq springs (30 - 62°C). The south region includes Wadi Bin Hammad (42 - 51°C), Wadi Addiraa (33°C), Al Barbaitah spring (38°C) and Afra springs (44 - 48°C) (Harahsheh, 2002; Sunna, 2004; Swarieh, 2000).

Interest in thermophilic organisms has been growing in recent years, few scattered microbial studies had been done on such springs in Jordan, but no studies describe thoroughly the diversity of bacteria and archaea in these hot springs especially at the molecular level. The purpose of the current study is to investigate the diversity of bacteria and archaea in some of these hot springs including using both culture-independent molecular approach (polymerase chain reaction) and culture- dependent approaches (conventional microbiology methods).

## MATERIALS AND METHODS

### Chemicals, media and reagents

All chemicals, media and reagents used in this study were purchased from Sigma Chemical Co. USA unless otherwise specified, as follows: D- Glucose (Chemlab Co. Burley. England), Yeast extract, PolyPeptone, Trypticase Soy Agar, Tryptone, Nutrient agar (Becton, Dickinson and Company. France), agarose (Promega Co. Madison. USA), all primers for PCR reactions (Alpha DNA Quebec. Montrial), PCR reagents Kit [10X PCR reaction buffer, 25 mM $MgCl_2$, 100 mM deoxy-nucleotides (dNTPs), Taq DNA polymerase enzyme and nuclease free water], 100 bp DNA ladder (Promega Co. Madison. USA), Proteinase K enzyme, Ribonuclease enzyme (RNase) (BDH Chemicals Ltd. UK), 1 kb DNA ladder (Fermentas. USA), PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc).

### Collection of samples

The samples were collected from Zarqa Ma'in springs, Zara springs, Ashounah well, Waggas well and Afra springs as follows:

1. Water samples from the spring water column: Two replicates were taken in sterile containers (3 liter bottles) from the source and canal for microbial cultivation and metagenomic DNA extraction.
2. Mat samples were collected in sterile falcon tubes with small volume of spring water; two sets of replicates from each site were collected, one set was stored at -20°C for later metagenomic DNA extraction. The other set was used for microbial enrichment and cultivation. All samples were stored in the ice box until shipped to the laboratory for analysis.

### Analysis of bacterial and archaeal communities

#### *Culture-dependent microbiological analysis*

***Cultivation, isolation and morphological characterization of thermophilic bacteria****:* Each mat sample (about 1 g) was suspended in 10 ml of sterile water, slightly agitated for 2 h at 100 rpm, then 100 µl of supernatants were poured and spread onto different thermal selective media plates for the isolation of thermophilic bacteria (Table 1). For water samples, 100 µl of each water sample were spread directly onto the selective plates. Plates were incubated at 50°C, except Zarqa Ma'in samples that were plated in duplicate and one set was incubated at 55°C while the other was incubated at 60°C. Different bacterial colonies that grew on the plates were purified by sub-culturing on the same selective thermophilic media.

Characterization of each bacterial isolate was performed morphologically according to colony color, size, elevation, margin and gram staining. Also, transmission electron microscopy (TEM) observation was done for some of the selected isolates.

***Determination of bacterial tolerance to grow at high temperatures***: Each bacterial isolate was cultivated at several temperatures (50, 55, 60, 70, 75, and 80°C) higher than the isolation temperature to determine their tolerance to grow at higher temperature and to determine the maximum growth temperature for each.

**Table 1.** Culture media used for growth and isolation of thermophilic microorganisms.

| Medium | Microorganism | Temperature of growth (°) | Reference |
|---|---|---|---|
| Tryptic Soy Agar | Thermophilic bacteria | 50 or above | Campbell et al.,2000 |
| Bacillus medium | Thermoacidophic bacteria | 50 or above | Lin et al., 1984 |
| Thermus medium | Thermophilic bacteria | 50 or above | Lin et al., 1984 |
| Castenholz TYE medium | Thermophilic bacteria | 50 or above | Lin et al., 1984 |
| Halophile medium (7, 12, 15 NaCl) | Thermohalophilc bacteria and archaea | 50 or above | Elshahed et al., 2004 |
| Nutrient agar | Thermophilic bacteria | 50 or above | Lin et al., 1984 |

***Culture-independent analysis (molecular methods)***

***Metagenomic DNA extraction from water and mat samples***: Metagenomic DNA was isolated directly from water samples from each hot spring site according to the protocol described by Delabre et al. (1998). Metagenomic DNA was extracted directly from mat samples using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc.), according to manufacture's instructions.

***Total genomic DNA extraction from pure cultures of bacterial isolates****:* Genomic DNA was isolated form each bacterial isolate as follows: One colony from each bacterial isolate culture was inoculated into 10 ml nutrient broth and incubated overnight at the same temperature that the isolate was initially grown. One ml from each of the nutrient broth culture was centrifuged at 15000 rpm for 15 min, the pellets were suspended in 0.5 ml lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 200 g/ml proteinase K. pH 8.5), and incubated at 55°C for 2 h, samples were then subjected to RNase treatment (1.5 mg/ml) for 30 min at 37°C. DNA was precipitated with equal volume of ice cold isopropanol and the pellets were washed with 75% ethanol, then dried and suspended in TE buffer. This DNA was used as template for PCR reaction.

***Polymerase chain reaction (PCR) amplification of bacterial and archaeal 16S rDNA sequences from metagenomic DNA extracted from water and mat samples***: Polymerase chain reaction amplification for the 16S rDNA sequences of bacterial domain was carried out according to Kanokratana et al. (2004) and the 16S rDNA sequences of archaeal domain was carried out according to Baker et al. (2003).

Polymerase chain reactions amplification for the 16S rDNA sequences of green sulfur, green nonsulfur bacteria and Heliobateria were carried out according to Achenbach et al. (2001). Polymerase chain reaction using pufM.557F and pufM.750R primers (Table 2) for amplification of the target sequence of M-subunit gene of the photosynthetic reaction center of purple phototrophic bacteria was carried out as described by Achenbach et al. (2001).

Polymerase chain reaction using MLf and MLr primers (Table 2) for amplification of the target sequence of -subunit gene of methyl coenzyme M reductase representing methanogenic archaea was carried out according to Juottonen et al. (2006).

Polymerase chain reaction amplification for the 16S rDNA gene sequences specific to the genus *Bacillus* was performed on genomic DNA isolated from each bacterial isolate according to Goto et al. (2000).

***Gel electrophoresis and photography***: Genomic DNA and the PCR amplification products were separated on 1% w/v agarose gels in 1X TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 ) at 100 V for I h using horizontal gel electrophoresis apparatus. Gels were stained with ethidium bromide (0.5 g/ml), and photographed by BioDocAnalyze (Biometra, Germany) . 100 bp or 1 kb DNA ladders were used as reference standards.

## RESULTS

### Morphological characterization of bacterial isolates

One hundred and thirty two different bacterial isolates (MAJ 1-MAJ 132) were cultivated and isolated from several hot springs in Jordan, these bacterial isolates were distributed as follow: (32) isolates (MAJ 1-MAJ 32) were isolated from Zarqa Ma'in spring site (1) including (8) isolates from water and 24 isolates from mat; 22 isolates (MAJ 33-MAJ 54) from Zarqa Ma'in spring site (2) including 8 isolates from water and 14 isolates from mat; 14 isolates (MAJ 55-MAJ 68) from Afra spring including 3 isolates from water and 11 isolates from mat; 9 isolates (MAJ 69-MAJ 77) from Zara spring site (1) including one single isolate from water and 8 isolates from mat; 5 isolates (MAJ 78-MAJ 82) from Zara spring site (2) including 3 isolates from water and 2 isolates from mat; 37 isolates (MAJ 82-MAJ 119) from Ashounah well including 17 isolates from water and 20 isolates from mat and 13 isolates (MAJ 120-MAJ 132) from Waggas well including 2 isolates from water and 11 isolates from mat.

The isolates grew well within 24 - 48 h on different media including nutrient agar, trypticase soy agar, thermus medium, Castenholz tryptone yeast extract, halophile medium with 7% and 12% NaCl and bacillus medium (Table 1).

Discrete bacterial colonies were characterized morphologically, bacterial colonies have different forms: punctiform, circular and irregular shape. While the color of the isolated colonies ranged from white, off white, beige, yellow, orange, or pink.

For gram staining reactions, the majority of the bacterial isolates (125 out of 132) were gram positive rods; they occur singly or in chains, while seven bacterial isolates were gram positive coccobacilli (MJ 59, MJ 61, MJ 78, MJ 81, MJ 90, MJ 91 and MJ 97).

Transmission Electron Microscopy (TEM) analysis of some bacterial isolates from hot springs (Figure 1)

**Table 2.** Primers used for detection of archaea and bacteria in PCR reactions.

| Amplicon length (bp) | Primer target sequence | Target microorganism group | Sequence (5 3 ) | Primer |
|---|---|---|---|---|
| 229 | *pufM* gene | Purple phototrophic bacteria | CGCACCTGGACTGGAC<br>CCCATGGTCCAGCGCCAGAA | pufM.557F<br>pufM.750R<br>(Achenbach et al., 2001) |
| 525 | 16S rRNA gene | Green sulfur bacteria | GGGGTTAAATCCATGTGCT<br>CAGTTCARTTAGAGTCC | GS.619F<br>GS.1144R<br>(Achenbach et al., 2001 |
| 384 | 16S rRNA gene | Green nonsulfur bacteria | TGCCTTAGCTCACGCGGTAA<br>GCAACGCATTGTCGTGGCCA | CFX.856F<br>CFX.1240R<br>(Achenbach et al., 2001) |
| 741 | 16S rRNA gene | *Heliobacteria* | TCTTCGGATTGTAAACCC<br>CCGGTCGTCCCGGGCA | HB.418F<br>HB.1159R<br>(Achenbach et al., 2001) |
| 1500 | 16S 16S rRNA gene rRNA gene | All bacteria (Universal) | AGAGTTTGATCCTGGCTCAG<br>GGTTACCTTGTTACGACTT | BSF8/20<br>REVB<br>(Kanokratana et al., 2004) |
| 650 | 16S rRNA gene | All Archaea (Universal) | GCYTAAAGSRICCGTAGC<br>TTMGGGGCATRCIKACCT | A571F<br>UA1204R<br>(Baker et al., 2003) |
| 320 | 16S rRNA gene | Genus *Bacillus* | TGTAAAACGACGGCCAGTGCC<br>TAATACATGCAAGTCGAGCG<br>CAGGAAACAGCTATGACCAC<br>TGCTGCCTCCCGTAGGAGT | *Bacillus F*<br>*Bacillus R (*Goto et al., 2000) |
| 470 | *mcrA* gene | *Methanogens* | GGTGGTGTMGGATTCAC<br>ACARTAYGCWACAGC<br>TTCATTGCRTAGTTWGGRTAGTT | MLf<br>MLr<br>(Juottonen et al., 2006) |

showed that they were either coccobacilli, long rods, or filamentous in shape, and occurred either single or in chains, and most of them are flagellated.

### Growth temperatures of bacterial isolates

All the bacterial isolates were thermophilic, they grew on different media at growth temperatures ranged from 50 to 75°C as indicated in Table 3, and no growth was obtained after 7 days at 80°C for any of the bacterial isolates.

According to bacterial growth at high temperatures, 19 bacterial isolates were able to grow at up to 75°C, 7 of these isolates (MAJ 1, MAJ 3, MAJ 4, MAJ 8, MAJ 10, MAJ 18, and MAJ 26) were from Zarqa Ma'in spring site (1), 6 isolates (MAJ 33, MAJ 37, MAJ 38, MAJ 41, MAJ 48, and MAJ 52) were from Zarqa Ma'in spring site (2), 4 isolates (MAJ 56, MAJ 60, MAJ 68, MAJ 71) were from Afra spring, one single isolate (MAJ 112) was from Ashounah well and another single isolate (MAJ 121) was from Waggas well. These bacterial colonies were circular and varied in size from small to medium, except single isolate (MAJ 68) which was large and irregular in shape.

Most of them had white to off white color, except isolates (MAJ 1 and MAJ 8) from Zarqa Ma'in spring site (1) and (MAJ 112) from Ashounah well which were yellow. For gram staining reactions, all of these isolates were gram positive rods.

### Determination of the pH range for bacterial growth

Bacterial growth from water and mat samples collected from hot springs was performed on several media; the pH of these media ranged from 7 to 8, except bacillus medium which was acidic with pH 3.5. So all the bacterial isolates have a pH for growth ranges from 7.0 to 8.0, except one single bacterial isolate (MAJ 11) from Zarqa Ma'in spring site (1) that grew on bacillus medium at pH 3.5, and the upper growth temperature for this isolate was up to 70°C, suggesting that it may be *Bacillus acidocaldarius*.

### Polymerase chain reaction (PCR) amplification of bacterial and archaeal 16S rDNA sequences from metagenomic DNA extracted from water and mat samples

Metagenomic DNA samples isolated from water and mat

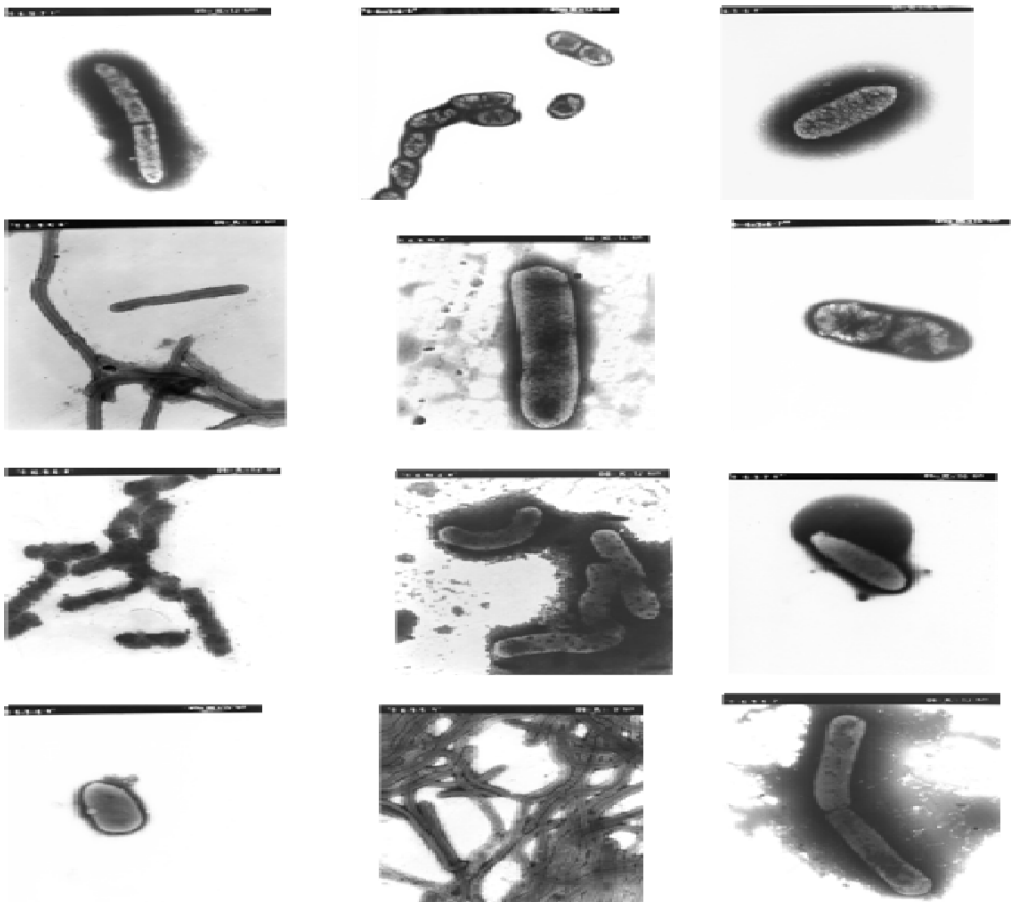

**Figure 1.** TEM photos of some selected bacterial isolates from hot springs.

**Table 3.** Bacterial growth tolerance to high temperatures.

| Growth at | | | | | | | Bacterial isolate |
|---|---|---|---|---|---|---|---|
| 80°C | 75˚C | 70˚C | 65˚C | 60˚C | 55˚C | 50˚C | and code # |
| - | + | + | + | + | + | + | MAJ 1, MAJ 3, MAJ 4, MAJ 8, MAJ10, MAJ 18, MAJ 26, MAJ 33, MAJ 37, MAJ 38, MAJ 41, MAJ 48, MAJ 52, MAJ 56, MAJ 60, MAJ 68, MAJ 71, MAJ112, MAJ121 |
| - | - | + | + | + | + | + | MAJ 2, MAJ 6, MAJ 11, MAJ 14, MAJ 20, MAJ 22, MAJ 29, MAJ 34, MAJ 35, MAJ 36, MAJ 40, MAJ 44, MAJ 44, MAJ 49, MAJ 65, MAJ69, MAJ 73, MAJ 82, MAJ 87, MAJ 94, MAJ 109 |
| - | - | - | + | + | + | + | MAJ 5, MAJ 7, MAJ 9, MAJ 12, MAJ 13, MAJ 15, MAJ 16, MAJ 17, MAJ 19, MAJ 21, MAJ 23, MAJ 24, MAJ 25, MAJ 27, MAJ 28, MAJ 30, MAJ 31, MAJ 39, MAJ 42, MAJ 43, MAJ 46, MAJ 47, MAJ 50, MAJ 51, MAJ 53, MAJ 55, MAJ 97, MAJ 101, MAJ 104, MAJ 108, MAJ 110, MAJ 113, MAJ 114, MAJ 116, MAJ 120 |

**Table 3.** Contd.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| - | - | - | - | + | + | + | MAJ 32, MAJ 45, MAJ, 54, MAJ 58, MAJ 59, MAJ 62, MAJ 66, MAJ 72, MAJ 74, MAJ 76, MAJ 79, MAJ 85, MAJ 86, MAJ 102, MAJ 107, MAJ 119 |
| - | - | - | - | - | + | + | MAJ 32, MAJ 45, MAJ 54, MAJ 61, MAJ 64, MAJ 67, MAJ 70, MAJ 75, MAJ 77, MAJ 78, MAJ 80, MAJ 81, MAJ 83, MAJ 84, MAJ 90, MAJ 92, MAJ 93, MAJ 95, MAJ 96, MAJ 98, MAJ 100, MAJ 103, MAJ 105, MAJ 106, MAJ 111, MAJ 117, MAJ 118, MAJ 122, MAJ 123, MAJ 124, MAJ 125, MAJ 126, MAJ 127, MAJ 128, MAJ 129, MAJ 132 |
| - | - | - | - | - | + | + | MAJ 57, MAJ 63, MAJ 88, MAJ 89, MAJ 91, MAJ 99, MAJ 129, MAJ 130, MAJ 131 |

(MAJ 1-MAJ 54) isolates from Ma'in springs, (MAJ 55-MAJ 68) isolates from Afra spring, (MAJ 69-MAJ 82) isolates from Zara springs, (MAJ 83-MAJ 119) isolates from Ashounah well, (MAJ 120-MAJ 132) isolates from Waggas well. (+) indicates bacterial growth at the indicated temperature within (24 - 8 h) incubation. (-) Indicates no bacterial growth at the indicated temperature even at 7 days incubation.

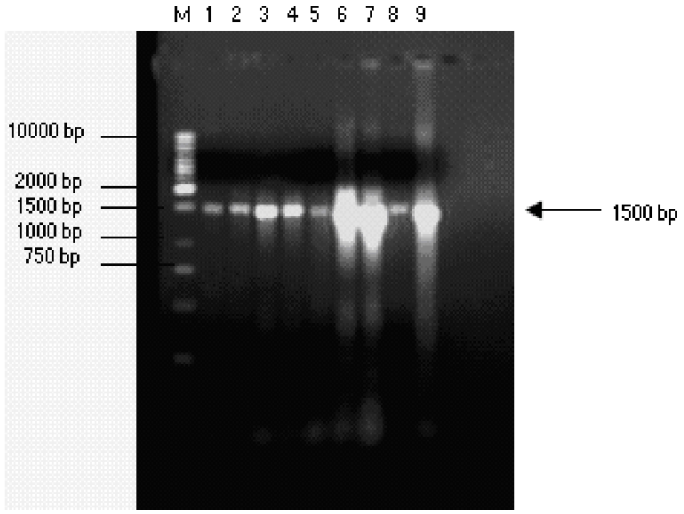


**Figure 2.** Representative picture of agarose gel electrophoresis of PCR amplification products of metagenomic DNA isolated from water samples using universal bacterial specific primer pair of 16S rDNA. Lane M, 1 kb DNA ladder; Lane 1, Zarqa Ma'in spring (1) source; Lane 2, Zarqa Ma'in spring (1) canal; Lane 3, Zarqa Ma'in spring(2) source; Lane 4, Zarqa Ma'in spring(2) canal; Lane 5, Afra spring source; Lane 6, Zara spring (1)source; Lane 7, Zara spring (2)source; Lane 8, Ashounah well source; Lane 9, Waggas well source.

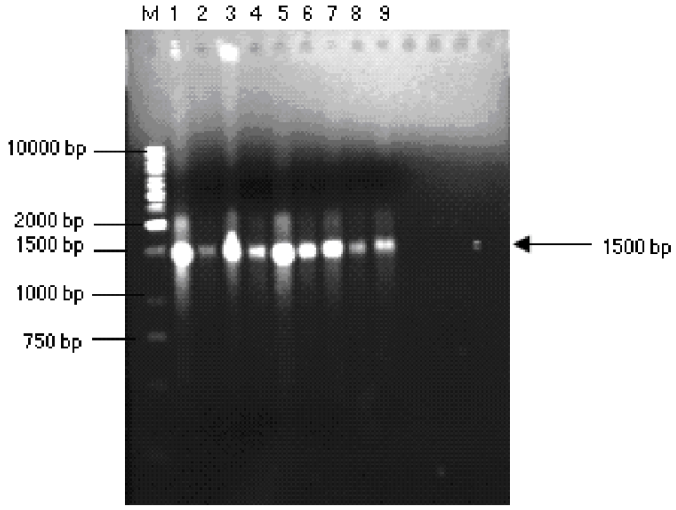


**Figure 3.** Representative picture of agarose gel electrophoresis of PCR amplification products of metagenomic DNA isolated from mat samples using universal bacterial specific primer pair of 16S rDNA. Lane M, 1 kb DNA ladder; Lane 1, Zarqa Ma'in spring (1) source; Lane 2, Zarqa Ma'in spring(1) canal; Lane 3, Zarqa Ma'in spring(2) source; Lane 4, Zarqa Ma'in spring(2) canal; Lane 5, Afra spring source; Lane 6, Zara spring (1)source; Lane 7, Zara spring (2)source; Lane 8, Ashounah well source; Lane 9, Waggas well source.

samples from each hot spring were subjected to PCR using bacterial 16S rDNA universal primer pair, all water and mat samples showed positive results for the presence of bacteria represented by the appearance of PCR amplification product of 1500 bp (Figure 2 and 3).

Metagenomic DNA samples isolated from water and mat samples from each hot spring were subjected to PCR using archaeal 16S rDNA universal primer pair, water samples from 4 sites includes: Zarqa Ma'in spring (1); source, Zarqa Ma'in spring (1); canal, Zarqa Ma'in

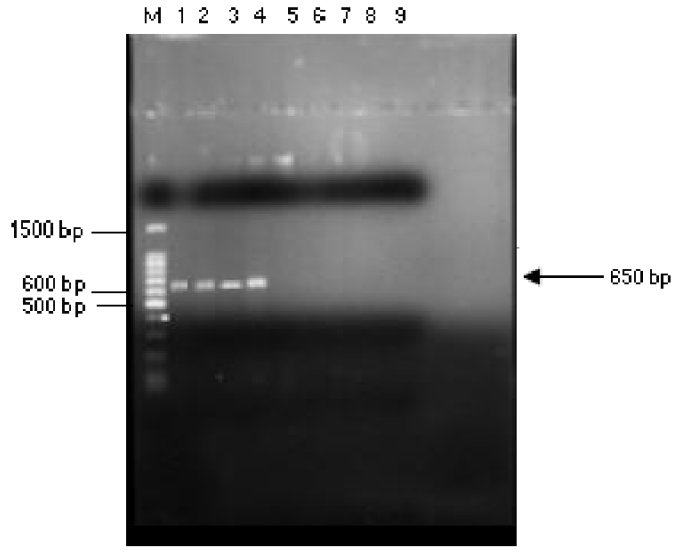


**Figure 4.** Representative picture of agarose gel electrophoresis of PCR amplification products of metagenomic DNA isolated from water samples using universal archaeal specific primer pair of 16S rDNA. Lane M, 100 bp DNA ladder; Lane 1, Zarqa Ma'in spring (1) source; Lane 2, Zarqa Ma'in spring(1) canal; Lane 3, Zarqa Ma'in spring(2) source; Lane 4, Zarqa Ma'in spring(2) canal; Lane 5, Afra spring source; Lane 6, Zara spring (1)source; Lane 7, Zara spring (2)source; Lane 8, Ashounah well source; Lane 9, Waggas well source.

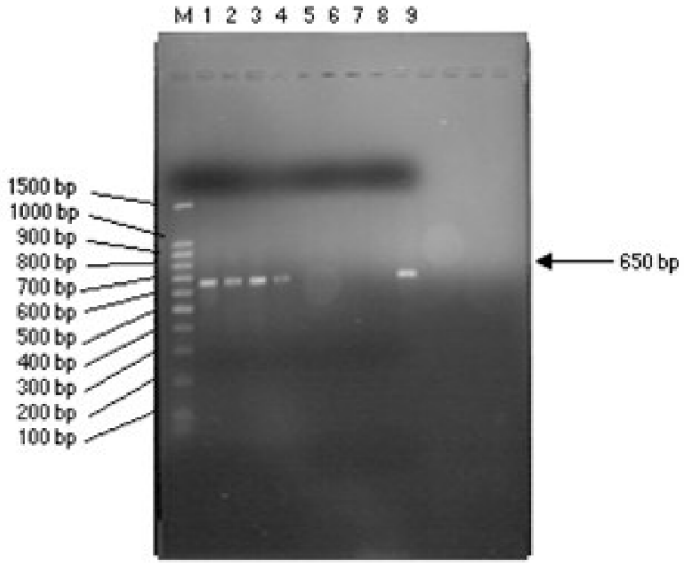


**Figure 5.** Representative picture of agarose gel electrophoresis of PCR amplification products of metagenomic DNA isolated from mat samples using universal archaeal specific primer pair of 16S rDNA. Lane M, 100 bp DNA ladder; Lane 1, Zarqa Ma'in spring (1) source; Lane 2, Zarqa Ma'in spring (1) canal; Lane 3, Zarqa Ma'in spring (2) source; Lane 4, Zarqa Ma'in spring (2) canal; Lane 5, Afra spring source; Lane 6, Zara spring (1) source; Lane 7, Zara spring (2) source; Lane 8, Ashounah well source; Lane 9, Waggas well source.

spring (2); source, Zarqa Ma'in spring (2); canal showed positive amplification represented by the appearance of PCR amplification product of 650 bp (Figure 4). Also, mat samples from 5 sites includes: Zarqa Ma'in spring (1); source, Zarqa Ma'in spring (1); canal, Zarqa Ma'in spring (2); source, Zarqa Ma'in spring (2); canal and Waggas well, source showed positive results represented by PCR amplification product of 650 bp size (Figure 5).

Metagenomic DNA samples isolated from water and mat samples from each hot spring were subjected to PCR using primer pair targeting 16S rDNA sequences of green sulfur bacteria, all PCR amplification reactions for water and mat samples showed negative results represented by absence of the expected amplified PCR product (525 bp).

Metagenomic DNA samples isolated from water and mat samples from each hot spring were subjected to PCR using primer pair targeting 16S rDNA sequences of green nonsulfur bacteria, mat samples from all hot springs sites (9 sites) showed positive results repre-sented by PCR amplification product of 384 bp (Figure 6). While water samples from all hot springs sites showed negative results (Figure not shown).

Metagenomic DNA samples isolated from water and mat samples from each hot spring were subjected to PCR using primer pair targeting 16S rDNA sequences of heliobacteria, all PCR amplification reactions for water and mat samples showed negative results represented by absence of the expected amplified PCR product (741 bp).

Metagenomic DNA samples isolated from water and mat samples from each hot spring were subjected to PCR using primer pair targeting M-subunit gene sequences of the photosynthetic reaction center of purple phototrophic bacteria, mat samples from 6 sites includes: Zarqa Ma'in spring (2); canal, Afra spring; source, Zara spring (1); source, Zara spring (2); source, and Waggas well; source showed positive results represented by the appearance of PCR amplification product of 229 bp (Figure 7). While water samples from all hot springs sites showed negative results.

Metagenomic DNA samples isolated from water and mat samples from each hot spring were subjected to PCR using primer pair targeting -subunit gene sequences of methyl coenzyme M reductase of the archaeal group methano-gens, all PCR amplification reactions for water and mat samples showed negative results represented by absence of the expected amplified PCR product (470 bp).

Genomic DNA was isolated from all bacterial isolates (MAJ 1-MAJ 132), each genomic DNA was subjected to PCR using primer pair targeting the 16S rDNA gene sequences specific to the genus *Bacillus*, 128 bacterial isolates (96.97% of total bacterial isolates) showed positive results, and gave the expected PCR amplification products of 320 bp (Figure 8), while only four isolates (MAJ 54, MAJ 61, MAJ 88 and MAJ 115) showed negative amplification.

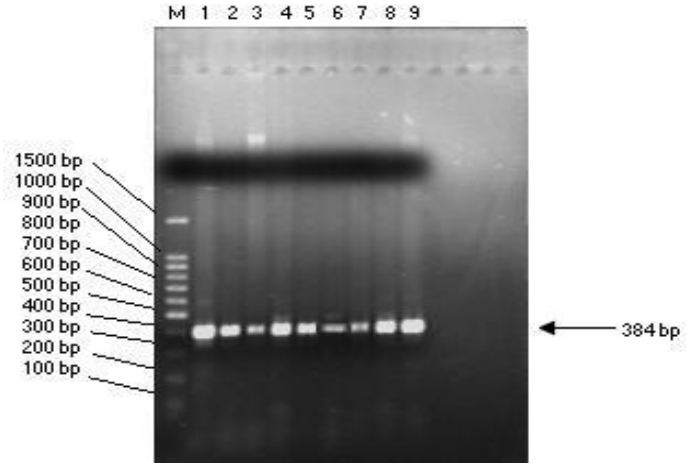


**Figure 6.** Representative picture of agarose gel electrophoresis of PCR amplification products of metagenomic DNA isolated from mat samples using primer pair specific to 16S rDNA of Green non sulfur bacteria. Lane M, 100 bp DNA ladder; Lane 1, Zarqa Ma'in spring (1) source; Lane 2, Zarqa Ma'in spring (1) canal; Lane 3, Zarqa Ma'in spring (2) source; Lane 4, Zarqa Ma'in spring (2) canal; Lane 5, Afra spring source; Lane 6, Zara spring (1) source; Lane 7, Zara spring (2) source; Lane 8, Ashounah well source; Lane 9, Waggas well source.

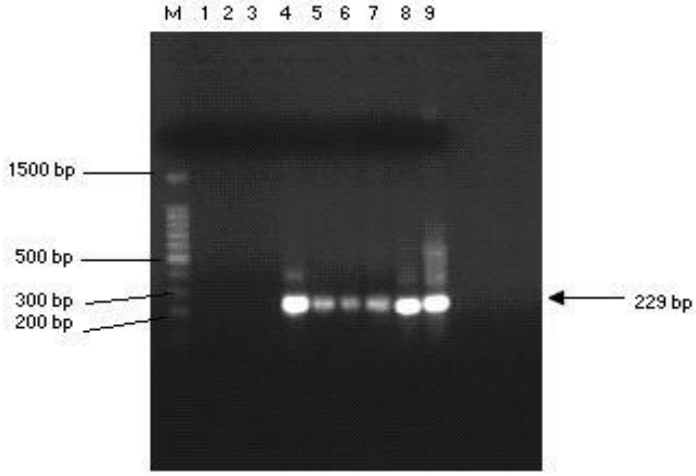


**Figure 7.** Representative picture of agarose gel electrophoresis of PCR amplification products of metagenomic DNA isolated from mat samples using primer pair specific to *pufM* gene fragment of Purple phototrophic bacteria. Lane M, 100 bp DNA ladder; Lane 1, Zarqa Ma'in spring(1) source; Lane 2, Zarqa Ma'in spring(1) canal; Lane 3, Zarqa Ma'in spring(2) source; Lane 4, Zarqa Ma'in spring(2) canal; Lane 5, Afra spring source; Lane 6, Zara spring (1)source; Lane 7, Zara spring (2)source; Lane 8, Ashounah well source; Lane 9, Waggas well source.

## DISCUSSION

In this study, the archaeal and bacterial community structure in mat and water samples from several hot springs in Jordan were investigated, the sites chosen for this study (Zarqa Ma'in spring (1), Zarqa Ma'in spring (2), Zara spring (1), Zara spring (2), Afra spring, Ashounah well and Waggas well) were of variable temperatures ranged from 49°C at Zara and Afra springs up to 63°C in Zarqa Ma'in spring (1), while they have pH ranges from 6.38 to 7.8.

The archaeal and bacterial diversity of these springs was investigated in the current study by both culture-dependent (conventional) and culture-independent molecular surveys (PCR), since typically, only a small fraction (<1%) of naturally occurring microorganisms is routinely cultivatable by standard techniques (Barns et al., 1996; Handelsman. 2004; Skirnisdottir et al., 2000). Such analysis enables access to more diversity than cultivation alone (Hobel et al., 2005).

Water and mat samples from Jordanian thermal springs were cultivated at high temperatures (50 or 55°C) on different selective media. A total of (132) bacterial isolates were recognized as thermophiles, since they were able to thrive at high temperatures above 50°C. Morphological analysis demonstrated that the majority of the thermophilic bacterial isolates from these springs were gram positive rods; in addition they showed diverse forms, colors, margins and shapes suggesting a great bacterial diversity at the species levels among the isolates.

Polymerase chain reaction indicated that the majority (128 out of 132) of isolates belong to the genus *Bacillus*. Previous studies had reported the dominancy of the genus *Bacillus* among hot springs, Bel'kova et al. (2005) reported the isolation of 39 thermophilic bacterial strains from water and bacterial mat from 73°C hot spring, most of them were assigned to the genus *Bacillus*.

Bacterial tolerance for growth at high temperature in this study indicated that 19 bacterial isolates identified within the genus *Bacillus* grew optimally at high temperature of up to 75°C. Blanc et al. (1997) reported the isolation of (750) heterotrophic, spore forming strains from hot compost (65°C), very few of these strains grew at temperatures above 65°C, and growth at 65°C was restricted to *Bacillus coagulans* and *Bacillus stearothermophilus.* In another study, Souza et al. (2001) described the isolation of aerobic, thermophilic, spore forming bacterial isolate that grew optimally at 55°C, and the upper temperature limit for growth was 70°C. The analysis of 16S rDNA gene sequences revealed that this isolate was phylogenetically closely related to members of the genus *Bacillus* group 5.

In this study, a unique thermoacidophilic Bacillus isolate (MAJ 11) which was able to thrive at low pH value (pH 3.5) and high temperature was purified and cultivated from Zarqa Ma'in spring. Previous study reported the cultivation of *B. acidocaldarius* on the same medium at 55°C (Angelov et al., 2006), by contrast the growth temperature range for our isolate was from 60 - 70°C and no growth was obtained at 55°C, which suggests and indicates also that a new and unique thermoacidophilic *Bacillus* strain was isolated.

In this study, bacterial 16S rDNA gene sequences were successfully amplified from all metagenomic DNA extracted from all water and mat samples from hot springs, indicating the presence of bacteria at these sites. Similar results of PCR amplification using the same primer pair had been reported by Kanokratana et al. (2004) when they used this primer pair to investigate bacterial diversity in Bor Khlueng hot spring in Thailand.

Archaeal DNA was amplified from Zarqa Ma'in springs and Waggas well, indicating the presence of archaeal domain in these springs, while the other springs (Afra, Zara and Ashounah) showed no archaeal DNA amplifications. Several studies reported archaeal DNA amplification from several hot springs using different primers (Kanokratana et al., 2004; Marteinsson et al. 2001). In contrast, another study was carried out at 45 - 90°C springs in Iceland, and no archaeal 16S rDNA gene sequences were found despite the use of various PCR conditions (Hobel et al., 2005). Methanogenic archaea was also investigated in this study using a primer pair targeting the *mcrA* gene sequences encoding the - subunit of methyl-coenzyme M reductase (MCR), which is essential for methane synthesis and potentially useful as biomarkers (Hallam et al., 2003; Juottonen et al., 2006), no PCR amplification product was obtained in any of water or mat samples from Jordanian hot springs despite trying different PCR conditions. Previous study had employed different *mcrA* primer pairs to describe methanogenic communities which resulted in the amplification of abroad selection of methanogens, and had a wider amplification range than the other primers (Juottonen et al., 2006). Several studies had reported the abundance of methanogens in hot springs microbial mat through sequencing of archaeal 16S rDNA sequences amplified from these sites (Barns et al., 1994; Delong et al., 1992).

Anoxygenic phototrophic bacteria inhabit a variety of extreme environments including thermal habitats (Roeselers et al., 2007). The diversity of anoxygenic phototrophs have been investigated using a variety of techniques, mainly PCR technique for the sensitive detection of specific genes from bacterial populations (Achenbach et al., 2001; Roeselers et al., 2007). In this study, a primer pair targeting 16S rDNA sequences of green sulfur bacteria was used, no PCR amplification product was obtained in any water or mat samples from Jordanian hot springs, suggesting the absence of this group of bacteria in those springs. Similar study was carried out using the same primer set and revealed two microbial mat samples from Yellowstone thermal springs (48 and 55°C) tested negative for green sulfur bacteria (Achenbach et al., 2001).

PCR analysis for all mat samples suggested the presence of green nonsulfur bacteria. Using the same primer set, green nonsulfur bacteria were detected in two hot springs from Yellowstone National Park (Achenbach et al., 2001). Several studies had reported the abundance of green nonsulfur bacteria in hot springs microbial mat through sequencing of bacterial 16S rDNA sequences amplified from these sites (Kanokratana et al., 2004; skirnisdottir et al., 2000).

The possibility of the presence of *Heliobacteriaceae* in Jordanian hot springs was also investigated in this study by PCR, but all water and mat samples from hot springs tested negative for the presence of *Heliobacteriaceae* family. By contrast *Heliobacterium modesticaldum,* a thermophilic heliobacterium, was isolated from microbial mats and volcanic soils of neutral to alkaline Yellowstone and Icelandic hot springs (Kimble et al., 1995), this organism showed positive PCR amplification with the same primer pair used by Achenbach et al. (2001).

Also in this study, another group of bacteria were detected (purple phototrophic bacteria) in mat samples from Zarqa Ma'in, Afra, Waggas and Zara springs in the PCR amplification. Previous studies also detected purple anoxyphototrophs in the New Pit spring sample from Yellowstone, and in all water and microbial mat samples tested from Lakes Fryxell and Hoare, Antractica using the same *PufM* primer set (Achenbach et al., 2001).

The environmental factors that directly influence hot spring microbial diversity most, remain unresolved, temperature has perhaps received the most attention, but other constraining factors such as pH and trace elements level may be key determinant of microbial community (Meyer-Dombard et al., 2005). The five hot springs sites targeted in this study were of variable temperature ranged between (49 - 63°C) and pH (6.38 - 7.8).

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