JJBS

Volume 19, Number 1,March 2026
ISSN 1995-6673
Pages 135 – 145
https://doi.org/10.54319/jjbs/190113

Jordan Journal of Biological Sciences

Isolation of *Tepidimonas taiwanensis* I1-1 and *Pseudoxanthomonas taiwanensis* NBRC 101072 from Jordanian Hot Springs: First Report

Miassar M. Al-Talib[1,2], Sara O. Hamdan[1,2], Mamoon M.D. Al-Rshaidat[1,2,*]

[1]*Department of Biological Sciences, School of Sciences, The University of Jordan, Amman, 11942, Jordan;* [2] *Laboratory for Molecular and Microbial Ecology (LaMME), The University of Jordan, Amman, 11942, Jordan*

Received: April 18, 2025; Revised: July 22, 2025; Accepted: August 21, 2025

Abstract

Thermophilic bacteria are defined by their ability to grow at high temperatures, where other life forms cannot exist. Jordan is rich in hot springs located along the Jordan Valley. This study aims to isolate and characterize thermophilic bacteria from Jordanian hot springs using enriched media containing all salts necessary for survival at high temperatures. Ten water samples were collected from hot springs in Ma'in, Zara, Dead Sea, North Shounah, and Mekhebh regions. Water samples were examined for physical and chemical properties. Two types of media containing salts and proteins necessary for the growth of thermophilic bacteria were used. These media are Thermus and Castenholz TYE media. Based on the morphological, microscopic, and molecular characterization results, 34 thermophilic bacterial isolates could be identified. Sequence analysis of the 16S rDNA and BLAST search of collected samples showed that *Bacillus licheniformis* is the most common bacterial isolate. Furthermore, data presented in this study report for the first time in Jordan the occurrence of *Tepidimonas taiwanensis* strain I1-1 and *Pseudoxanthomonas taiwanensis* strain NBRC 101072 in Jordanian hot springs. The phylogenetic relationship between the isolates and the referenced bacteria shows a close relationship with the *Anoxybacillus flavithermus* subsp. *flavithermus* strain DSM 2641, *Flavobacterium thermophilum* strain G-21, *Anoxybacillus rupiensis* strain ATCC BAA-2555, *Anoxybacillus kestanbolensis* strain K1, and *Anoxybacillus contaminans* strain R-16222. The study concluded that the isolation of *Tepidimonas taiwanensis* strain I1-1 and *Pseudoxanthomonas taiwanensis* strain NBRC 101072 for the first time from hot springs in Jordan may open the door for further research to investigate the activities and benefits of these bacteria.

Keywords: Bacteria, Hot Springs, Jordan, Phylogenetic tree, Thermophilic.

1. Introduction

Thermophilic bacteria are classified under the third domain of life (Archaea) because of their unique ability to survive at high temperatures (optimum growth temperature of 50°C or higher), unlike their counterparts from other groups of bacteria (Hugenholtz et al., 1998; Crosby et al., 2019; Fongaro et al., 2020; and Shakya et al., 2025).

Hot springs are unique areas characterized by high temperatures and a great natural environmental diversity (Yohandini et al.,2015). Hot springs worldwide attract researchers to investigate the industrial benefits of bioproducts of the thermophilic microorganisms inhabiting them (Malkawi and Al-Omari, 2010; Wolella and Tilahun, 2020, and Burkhardt et al., 2024)

As highlighted in recent reviews, researchers study thermophilic bacteria to discover new products that support biotechnology and medicine, especially enzymes with exceptional stability under industrial conditions (Canganella and Wiegel, 2014; Mehta et al., 2016; and Shomali & Danish-Daniel, 2024). Microorganisms and their thermostable enzymes (thermozymes) have emerged as pivotal tools in high-temperature industrial bioprocesses. According to Li et al. (2025), these biological catalysts not only maintain activity under extreme thermal conditions but also demonstrate remarkable efficacy in biomass conversion, polymer degradation, and pollutant detoxification. Recent integration of AI-based enzyme engineering and high-throughput screening has significantly enhanced the efficiency of thermozymes in biotechnological workflows, offering cost-effective and environmentally sustainable solutions for large-scale industries.

Supporting this, a recent metagenomic study conducted in Guizhou Province, China, revealed that hot springs harbor taxonomically and functionally diverse thermophilic bacterial communities, dominated by *Pseudomonadota* and *Bacillota*. Functional predictions indicated that these communities possess robust metabolic pathways for amino acid and carbohydrate metabolism, as well as genetic traits related to stress adaptation (Chen et al., 2025). These findings emphasize the ecological and industrial significance of hot spring microbiota and

* Corresponding author. e-mail: m.rshaidat@ju.edu.jo.

** **Abbreviation**s: BLAST: Basic Local Alignment Search Tool, MEGA11: Molecular Evolutionary Genetics Analysis version 11, PCR: Polymerase Chain Reaction, rDNA: Ribosomal Deoxyribonucleic Acid.

reinforce the value of exploring extreme environments for novel microbial resources.

The investigation and isolation of thermophilic bacteria from hot springs in Jordan started early in the eighties, with the isolation of *Bacillus licheniformis*, *Thermomonas hydrothermalis*, and *Caldimonaas hydrothermale* (Malkawi and Al-Omari, 2010; Mohammad et al., 2017; and Obeidat and Al-Shomali, 2023). Jordan has several hot springs along the Dead Sea area, the lowest land area on Earth. (Schäffer and Sass,

2014). There are about 200 thermal provinces in Jordan, the most famous of which is the Ma'in Hot Spring, frequently visited for tourism and therapeutic purposes (Schäffer and Sass, 2014; Ayadi et al., 2023). In this study, samples were collected from only four selected hot springs.

The four hot springs selected for this study are Ma'in Hot Spring, Zara Dead Sea, North Shounah, and Makhebh (Figure 1).

Figure 1. Map of Jordan displaying the specific hot spring sites included in this study. Water samples were collected from the marked locations, which are indicated with red asterisks.

Several reports have outlined the types of thermophilic microorganisms prevalent in Jordanian hot springs (Malkawi and Al-Omari, 2010; Yohandini et al., 2015; Mohammad et al., 2017; Obeidat and Al-Shomali, 2023), but still, plenty of information has not been explored. Accurate identification techniques depend on molecular and biological methods, especially the polymerase chain reaction (PCR) for the detection and classification of thermophilic species. To obtain a pure culture of bacteria, culture-dependent media that help isolate a pure single colony for further identification must be used (Malkawi and Al-Omari, 2010; Pinevich et al., 2018 and Kapinusova et al., 2023). Numerous types of dependent culture media have been previously used, such as Bacillus media, Castenhoiz TYE media, Tryptic Soy Agar media, Thermus media, and Halophile media The progress in molecular techniques, such as the amplification of the 16S rDNA gene, has allowed the simultaneous detection of many DNA sequences of different groups of microorganisms, and now a huge number of studies are also reducing phylogenic relationships among thermophilic bacteria (Ash et al., 1991; Mouné et al., 2003; Clarridge, 2004; Alsanie et al., 2018 and Zhao et al., 2025).

This study aims to isolate thermophilic bacteria from Jordanian hot springs using environmentally relevant enrichment media and to compare their DNA sequences with those of previously reported thermophiles.

Understanding the diversity and features of thermophilic bacteria from Jordanian hot springs helps fill regional gaps in microbial ecology, especially in underexplored geothermal environments in the Middle East. Additionally, isolating and studying these microorganisms can lead to the discovery of new thermostable enzymes and bioactive compounds with potential industrial, medical, and environmental uses. This research lays the groundwork for future advances in biotechnology and microbial resource preservation, particularly as interest grows in sustainable, bio-based solutions from extremophiles.

2. Materials and Methods

2.1. Sample collection

Ten water samples were collected from four different hot springs in Jordan, namely: Ma'in, Zara Dead Sea, North Shounah (Al-Hema), and Mekhebh hot springs, using sterile thermal glass containers. Care was taken during transportation to the laboratory to minimize evaporation effects and maintain sample integrity. Three replicates were collected from each springhead sampling location.

2.2. Chemical analysis of water samples

The hydrogen ion concentration (pH), electrical conductivity (EC), and temperature were measured on-site using a pH meter, portable EC meter (Trans Instruments, HC3010; Singapore), respectively. Chemical analyses were performed using a spectrophotometer (Spectro UV-VIS Double Beam PC, 8 Scanning Auto Cell, UVD, Labomed, Inc., Los Angeles, USA) in the facilities of the Jordan Valley Authority.

The chemical analysis for cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}), potassium (K^{+}), iron (Fe^{2+}), and anions such as nitrate (NO_3^{-}), and sulfate (SO_4^{2}) were conducted in the laboratory using an atomic absorption spectrophotometer.

The chloride (Cl^{-}) and bicarbonate (HCO_3^{-}) concentrations were determined using the titration method (Al-Khashman et al., 2017; Mohammad et al., 2017; and Edet et al., 2024).

2.3. Cultivation of water samples

For the isolation of thermophilic bacteria from the collected samples, the culture-dependent method using enrichment media and incubation, and high temperatures described by Malkawi and Al-Omari (2010) was adopted, with some modifications. Specifically, the culture media were prepared using filtered hot spring water collected from the sampling sites instead of distilled water. In addition, all enrichment cultures were incubated at a fixed temperature of 55 °C. Two types of culture media were used in this study, namely: Thermus medium and Castenholz TYE (tryptone yeast extract) medium.

2.3.1. Thermus medium preparation

Thermus medium is composed of 5 g tryptone, 5 g yeast extract, 1 g sodium chloride, 0.2 g magnesium sulfate ($MgSO_4$), 2 g potassium phosphate (K_2HPO_4), 0.1 g calcium chloride ($CaCl_2$), 0.1 g ferric chloride ($FeCl_3$),

0.1 g sodium thioglycolate, 0.02 g nitrilotriacetic acid (NTA), and 0.02 g ethylenediaminetetraacetic acid (EDTA). All components were dissolved in one liter of filtered hot spring water in bottles. The pH was adjusted to 7.0 using an adjustment solution (1 M NaOH or 1 N HCl). For solid medium preparation, 15 g of agar was added. The media were then sterilized using an autoclave at 121 °C, 15 psi for 20 minutes (Kumar et al., 2025).

After sterilization, the bottles were cooled in a water bath to approximately 50 °C and poured into sterile petri dishes. To ensure the absence of contamination before inoculation, the solidified plates were incubated overnight at 55 °C.

2.3.2. Castenholz TYE medium preparation

The Castenholz Tryptone-Yeast Extract (TYE) medium was used according to Castenholz, (1969). The medium consists of salts with one part (1%) TYE (Tryptone and Yeast Extract) and four parts distilled water. The medium was heated and mixed with TYE solution and autoclaved at 121 °C. The salts were added aseptically, and the final pH of the medium was adjusted to 7.6. Agar plates were prepared by adding 3% agar before autoclaving

Water samples from hot springs were inoculated onto Petri dishes using two selective media and incubated at 55°C to promote the growth of thermophilic bacteria. Subculturing was repeated by transferring isolated colonies onto fresh plates until 34 pure, single colonies were obtained. All chemicals and reagents used for the preparation of culture media in this study were obtained from Bio Basic Inc. (Markham, Ontario, Canada).

2.4. Isolation and characterization of isolates

Thermophilic bacteria were enriched on previously prepared agar media by incubating the samples at 55°C. The colonies were further classified according to their shape and size. Some of the colonies were then stained using Gram staining. Colony morphology was assessed after 48 hours of incubation at 55°C on agar media. Morphological characterization included visual evaluation of colony color, shape, edge characteristics, surface texture, and approximate size. Colonies were generally circular with entire or slightly undulate margins and smooth to slightly mucoid surfaces. Colors varied among isolates, including white, cream, cream-white, pale yellow, and yellow. Colony diameters were visually estimated to range between 1 and 3 mm. Gram staining was performed following standard microbiological procedures to differentiate isolates based on their cell wall characteristics. Freshly grown colonies were smeared onto glass slides, heat-fixed, and sequentially treated with crystal violet, iodine solution, decolorizer ethanol, and counterstained with safranin. The stained slides were examined under a light microscope at 1000× magnification using oil immersion. Observations included Gram reaction and cellular morphology. The Gram staining reagents were obtained from Millipore Sigma (USA), kit number 77730-1KT-F.

2.5. 16S rDNA amplification and sequencing

A single colony from the agar plate was picked up with an inoculation loop and cultured overnight at 55 °C on Castenholz TYE broth medium.

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA, Cat. No. A1620) according to the manufacturer's instructions, and the concentrations of extracted DNA were determined using a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). The 16S rDNA gene was used for bacterial identification, and it was amplified by PCR using the domain bacterial-specific primer 27F (5'- AGA GTT TGA TCM TCG CTC AG-3') and universal primers 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3') (Mohammad et al. 2017). The reaction mixture (25 µL) consisted of 1X PCR reaction buffer (2.5 µL), 2.5mM $MgCl_2$ (2.5 µL), 0.2mM deoxynucleoside triphosphate (0.5 µL), 0.1mM of each primer (0.5 µL), 1 U Taq DNA polymerase (0.5 µL), 1 µL of 50ng genomic DNA, and 17 µL nuclease-free water. All PCR components were obtained from the Taq DNA Polymerase PCR Buffer kit (Cat. No. 18067-017, Thermo Fisher Scientific, USA).

The PCR reaction was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA). The amplification conditions were as follows: denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel, visualized under a UV transilluminator (Gel Doc 200, BIO-RAD, USA), and photographed with the gel documentation system (Gel Doc 200, BIO-RAD, USA) after staining with ethidium bromide (0.5 µg/mL). One kb DNA ladder (GeneRuler 1 kb DNA ladder, SM0313, ThermoFisher Scientific, MA, USA) was used as a marker to determine the size of the amplified fragments. Amplified 16S rDNA gene fragments were sequenced using commercial services at Macrogen Inc. (Korea). The phylogenetic tree was constructed using the neighbor-joining method, based on distance matrix analysis performed with Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) software, and 1000 bootstrap replications were used. Sequence comparisons and database searches were conducted using the BLAST algorithm (Tamura et al., 2021).

3. Results

3.1. Physical and chemical analysis of water samples

Ten water samples were collected from four hot spring locations in Jordan: Ma'in Hot Spring, Zara Dead Sea, North Shounah (Al-Hemma), and Mekhebh Hot Spring. The water temperature was recorded between 40.0 and 55.0°Cwhich supports the growth of thermophilic bacteria. Table 1 summarizes all physical characteristics of the collected samples.

Table 1. Physical characteristics of water samples from geothermal sites in Jordan, including collection locations, electrical conductivity (EC), temperature, and pH.

Collection site	Geographic Coordinate	Sample Code	Electrical Conductivity (mS/cm^2)	Water Temperature (°C)	pH
Ma'in 1	31.608829 35.611118	MN (1,213,4,5,6) S1	2.78	40.0	8.19
Ma'in 2	31.58333 N 35.73333E	MN (7,8,9,10) S2	2.90	54.0	7.29
Ma'in 3	31.3634.6N 35.3647.8E	MN (11,12,13) S3	3.21	50.0	8.19
Zara Dead Sea	31.3550.1N 35.3400.8E	ZARA (1,2,3)	1.64	48.0	7.69
Shounah 1	32.8124620 35.6169760	SH (9,10) S1	1.42	48.0	8.20
Shounah 2	32.6151040 35.6227250	SH (1,3) S2	0.956	46.2	7.32
Shounah 3	32.6130150 35.6166320	SH (2,4,5,6,7,8,11) S3	1.46	55.0	7.45
Mekhebh 1	32.7046230 35.6837450	MK (1,3,4) S1	1.53	41.0	7.51
Mekhebh 2	32.7045260 35.6838080	MK (2) S2	1.53	40.3	7.82
Mekhebh 3	32.7045330 35.6837810	MK (5,6,7) S3	1.54	41.6	7.58

The four water sources are different in terms of mineral content. For example, chemical analysis of the water sample obtained from Ma'in hot spring water showed that it contains moderate bicarbonate (HCO_3^-) and low nitrate (NO_3^-) but high calcium (Ca^{2+}), sodium (Na^+), sulfate (SO_4^{2-}), and chloride (Cl^-). On the other hand, the water sample collected from Zara had the lowest nitrate (NO_3^-) concentration of all water samples, and it contained a slightly reduced bicarbonate (HCO_3^-) concentration than that found in Ma'in hot spring. It is also characterized by elevated levels of salt, chloride, and considerably larger amounts of calcium and potassium. The water sample obtained from North Shounah had the highest levels of magnesium, bicarbonate, and moderate sodium, less calcium, and a slightly higher nitrate. Table 2 summarizes ion concentrations (ppm) for each site.

Table 2: Chemical analysis of water samples collected from selected hot springs in Jordan.

Water source	Mg^{2+} ppm	Ca^{2+} ppm	Na^+ ppm	K^+ ppm	NO_3^- ppm	SO_4^{2-} ppm	Cl^- ppm	HCO_3^- ppm
Ma'in	35.66	135.20	176.3	32.20	0.51	293.64	298.65	202.40
Zara	25.99	110.0	290.0	40.60	0.32	296.86	428.14	183.02
North Shounah	88.43	55.70	161.0	9.40	0.63	295.98	219.45	300.16
Mekhebh	39.87	50.0	51.70	2.20	0.73	28.29	103.01	292.84

Note: Ca – Calcium, Na – Sodium, SO_4^{2-} – Sulfate, Cl^- – Chloride, HCO_3^- – Bicarbonate, NO_3^- – Nitrate, Mg^{2+} – Magnesium, K^+ – Potassium, Fe^{2+} – Iron

Finally, Mekhebh water contains low concentrations of Na^+ and Cl^- and is characterized by a generally lower concentration of other ions except NO_3^-. These variances suggest that each water source has a unique mineral content, which might affect its suitability for a given purpose and the microbes it harbors. For instance, the high salt concentration in Ma'in and Zara hot springs may promote the growth and development of halophilic microbes, whereas differing profiles in North Shounah and Mekhebh suggest the ability to support various bacterial types based on their metabolic demand and threshold level of minerals. Water samples from all sources had iron levels less than 1 µg/L.

3.2. Phenotypic characterization of bacterial isolates

The bacterial strains collected from different hot springs displayed variable Gram staining, cell morphology, and colony pigmentation. Gram-positive bacilli were the most dominant, including *Anoxybacillus* and *Bacillus* species. These strains, derived from the hot spring samples, were metabolically active under either facultatively aerobic or anaerobic conditions with yellow-cream-colored colonies. On the other hand, Gram-negative bacilli, particularly *Flavobacterium* and *Sphingobacterium* species, grew aerobically and produced whitish to pale-yellow colonies. The variation of the Gram staining coupled with the coloration of the colonies indicates the existence of a variety of microorganisms thriving at the elevated temperatures of hot springs, as shown in Table 3.

Table 3. Gram staining results, cell morphology, and phenotypic characteristics of bacterial isolates obtained from hot springs in Jordan.

Tube No.	Location	Species	Gram stain	Colony Shape	Oxygen requirements	Colony Color
1	Ma'in 1	*Anoxybacillus flavithermus* subsp. flavithermus strain DSM 2641	+ve	Rod	Facultatively aerobic	Yellow
2	Ma'in 1	*Tepidimonas taiwanensis* strain I1-	-ve	Rod	Aerobic	Cream white
3	Ma'in 1	*Tepidimonas taiwanensis* strain I1-1	-ve	Rod	Aerobic	Cream white
4	Ma'in 1	*Tepidimonas taiwanensis* strain I1-1	-ve	Rod	Aerobic	Cream white
5	Ma'in 1	*[Flavobacterium] thermophilum* strain G-21	-ve	Rod	Aerobic	White
6	Ma'in 1	*Anoxybacillus flavithermus* subsp. flavithermus strain DSM 2641	+ve	Rod	Facultatively aerobic	Yellow
7	Ma'in 2	*Anoxybacillus rupiensis* strain ATCC BAA-2555	+ve	Rod	Aerobic	Cream white
8	Ma'in 2	*Flavobacterium thermophilum* strain G-21	-ve	Rod	Aerobic	White
9	Ma'in 2	*Anoxybacillus flavithermus* subsp. flavithermus strain DSM 2641	+ve	Rod	Facultatively aerobic	Yellow
10	Ma'in 2	Anoxybacillus kestanbolensis strain K1	+ve	Rod	Aerobic	Cream white
11	Ma'in 3	*Anoxybacillus contaminans* strain R-16222	+ve	Rod	Facultatively anaerobic	Cream-colored
12	Ma'in3	*Anoxybacillus rupiensis* strain ATCC BAA-2555	+ve	Rod	Aerobic	Cream white
13	Ma'in 3	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
14	ZARA	*Flavobacterium thermophilum* strain G-21	-ve	Rod	Aerobic	White
15	ZARA	*Geobacillus stearothermophilus* strain BGSC 9A20	+ve	Rod	Anaerobe	White
16	ZARA	*Flavobacterium thermophilum* strain G-21	-ve	Rod	Aerobic	White
17	Shounah 1	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
18	Shounah 1	*Sphingobacterium thermophilum* strain CKTN2	-ve	Rod	Aerobic	Pale yellow
19	Shounah 1	*Sphingobacterium thermophilum* strain CKTN2	-ve	Rod	Aerobic	Pale yellow
20	Shounah2	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
21	Shounah3	*Flavobacterium thermophilum* strain G-21	-ve	Rod	Aerobic	White
22	Shounah3	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
23	Shounah3	*[Flavobacterium] thermophilum* strain G-21	-ve	Rod	Aerobic	White
24	Shounah3	*Pseudoxanthomonas taiwanensis* strain NBRC 101072	-ve	Rod	Aerobic	Yellow
25	Shounah3	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
26	Shounah 3	*[Flavobacterium] thermophilum* strain G-21	-ve	Rod	Aerobic	White
27	Shounah 3	*Anoxybacillus flavithermus* subsp. flavithermus strain DSM 2641	+ve	Rod	Facultatively aerobic	Yellow
28	Mekhebh 1	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
29	Mekhebh1	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
30	Mekhebh1	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
31	Mekhebh 2	*Anoxybacillus contaminans* strain R-16222	+ve	Rod	Facultatively anaerobic	Cream-colored
32	Mekhebh 3	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
33	Mekhebh 3	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored
34	Mekhebh 3	*Bacillus licheniformis* strain DSM 13	+ve	Rod	Facultative anaerobic	Cream-colored

3.3. Molecular characterization of the isolates

The PCR products of 16S rDNA of thirty-four (34) thermophilic bacterial isolates were analyzed by electrophoresis on 1% agarose gel (Figure 2) and sequenced. The sequences were used for the identification and phylogenetic analysis of these isolates.

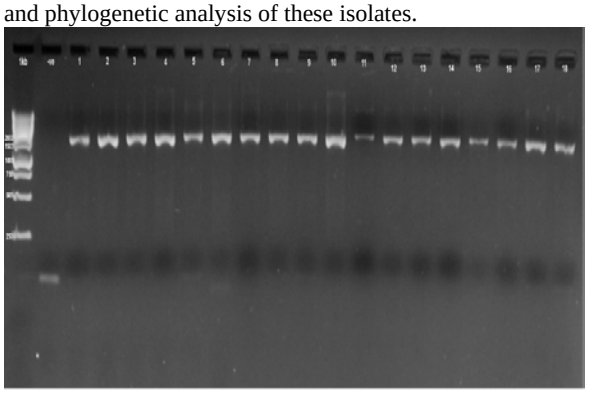

Figure 2. Agarose gel electrophoresis of PCR-amplified 16S rDNA gene fragments from bacterial isolates using the universal primer pair 27F/1492R. Lanes 1–18: PCR products from individual bacterial isolates. Lane 1 kb: DNA ladder (1 kb) used as a molecular size marker. Lane -ve: negative control (no template DNA). The expected amplicon size is approximately ~1.5 kb.

3.4. Phylogenetic analysis

The 16S rDNA sequences of the thirty-four thermophilic bacterial isolates were aligned with known thermophilic reference sequences from GenBank using the BLAST program (Figure 3). These reference sequences are represented by the following accession numbers: NR_104891 (*Flavobacterium thermophilum* **)**, NR_115109 (*Anoxybacillus gonensis*), NR_026516 (*Anoxybacillus flavithermus* **)**, NR_180406 (*Anoxybacillus kestanbolensis***)**, NR_116097 (*Anoxybacillus mongoliensis***)**, NR_117229 (*Anoxybacillus eryuanensis***)**, NR_029006 (*Anoxybacteroides contaminans***)**, NR_024818 (*Anoxybacteroides voinovskiense*), NR_026515 (*Aeribacillus pallidus*), NR_115284 *(Geobacillus stearothermophilus)*, NR_119305 (*Geobacillus thermocatenulatus*), NR_118996 *(Bacillus licheniformis*), NR_113993 (*Bacillus sonorensis*), NR_113974 (*Pseudoxanthomonas taiwanensis* strain NBRC 101072), NR_025198 (*Pseudoxanthomonas taiwanensis* strain CB-226), NR_113984 (*Pseudoxanthomonas daejeonensis* strain NBRC 101159*)*, NR_042418 (*Tepidimonas thermarum*), NR_179191 (*Tepidimonas arfidensis*), NR_180139 (*Tepidimonas charontis*), NR_043227 (*Tepidimonas taiwanensis* strain I1-1), and NR_043433 (*Sulfuracidifex metallicus*).

The phylogenetic relationships showed that isolates MN1 S1, MN6 S1, MN9 S1, and SH11 S3 were closely related to *Anoxybacillus flavithermus* subsp. *flavithermus* strain DSM 2641, while isolates MN2 S1, MN3 S1, and MN4 S1 were closely related to *Tepidimonas taiwanensis* strain I1. Six isolates including MN5 S1, MN8 S1, ZARA1, ZARA3, SH3 S3, and SH8 S3 were closely related to *Flavobacterium thermophilum*strain G-21. Two isolates, MN7 S2 and MN12 S3, were closely related to *Anoxybacillus rupiensis* strain ATCC BAA-2555. In addition, isolates MN10 S2 (*Anoxybacillus skestanbolensis* strain K1), MK2 S2, MN11 S2, and MN11 S3 were found to be closely related to *Anoxybacillus contaminans* strain R-16222. *Bacillus licheniformis* strain DSM 13 is the most common Bacillus species found in the hot spring water. Sequence analysis showed that isolates MK1 S1, MK3 S1, MK4 S1, MK5 S3, MK6 S3, MK7 S3, SH1 S2, SH3 S2, SH4 S3, SH7 S3, and MN13 S3 are closely related to *T*. In addition, isolate SH2 S3 was found to be related to *Flavobacterium thermophilum* strain G-21, isolate SH6 S3 is related to *Pseudoxanthomonastaiwanensis*strain NBRC 101072, and isolate ZARA2 is closely related to *Geobacillus stearothermophilus* strain BGSC 9A20.

Analysis of the 16S rDNA sequences of isolates obtained from Ma'in, North Shounah, Mekhebh, and Zara formed distinct clades within the genera *Anoxybacillus*, *Geobacillus*, *Pseudoxanthomonas*, and *Tepidimonas* with a bootstrap support >70%. Ma'inisolates are clustered with *A.flavithermus* and *Anoxybacillus kestanbolensis* with bootstrap values of 99% and 95% respectively, indicating a close relationship with these thermophiles. Zara and NorthShounah isolates are grouped with *Flavobacterium thermophilum*and *Anoxybacillus* species with 100% bootstrap values. It is worth mentioning that *T.taiwanensis*was isolated for the first time in Jordan from Ma'in hot spring, and it formed a clade with other *Tepidimonas* species with a 98% bootstrap value. This is the first report of *T. taiwanensis* in Jordan, indicating local adaptation of this thermophilic bacterium in the Ma'in hot spring.

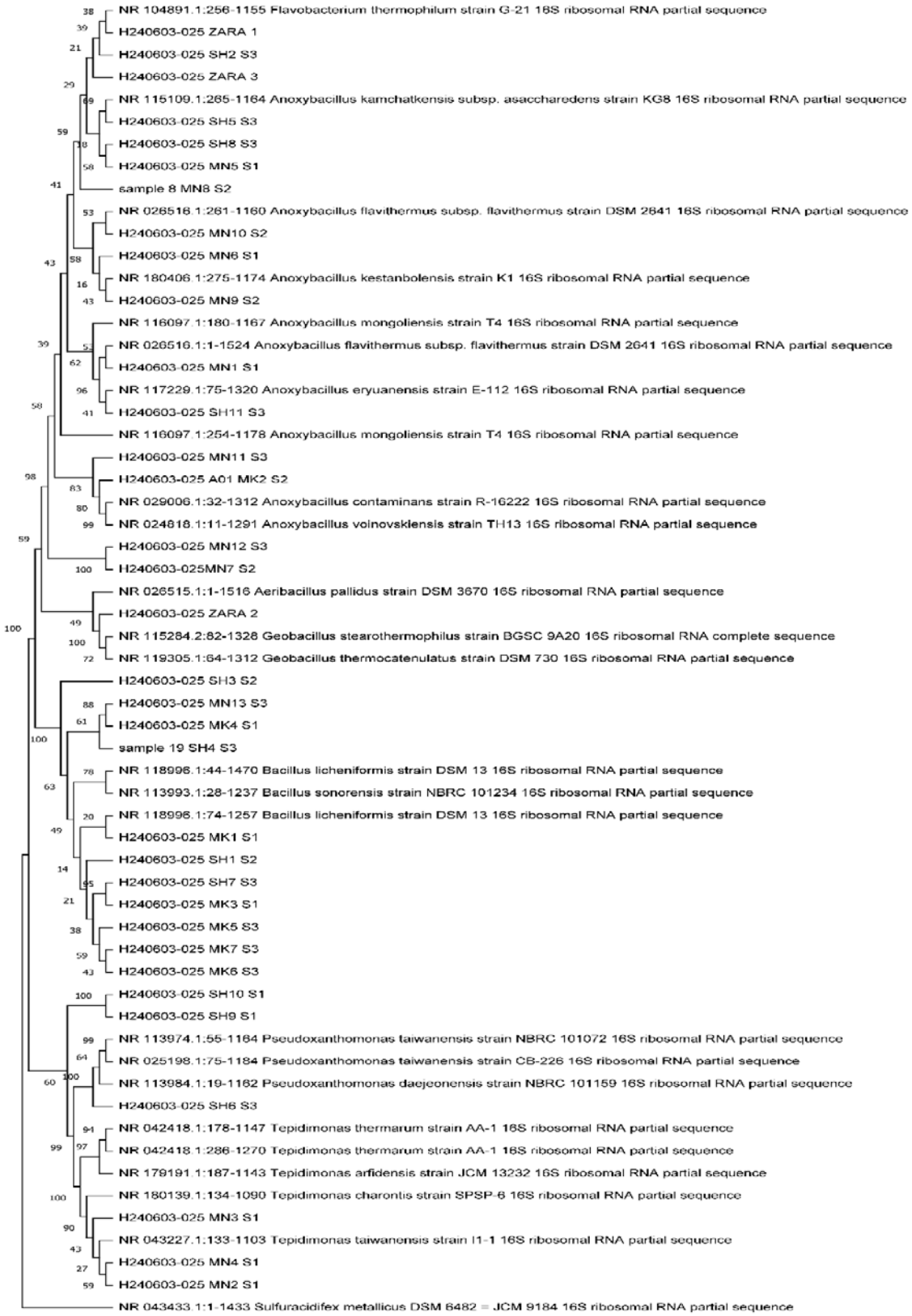

Figure 3. A phylogenetic tree illustrates the evolutionary relationships among bacterial isolates based on 16S rDNA gene sequences. The tree was constructed using the Neighbor-Joining (NJ) method with the p-distance model to calculate genetic distances. Statistical support for the branching pattern was assessed by bootstrap analysis with 1000 replications, and the bootstrap values (%) are indicated at each node. Higher bootstrap values indicate stronger support for the corresponding clades. *Sulfuracidifex metallicus* (DSM 6482 = JCM 9184) was included as an outgroup to root the tree and provide evolutionary context. The analysis was performed using MEGA version 11 (Tamura, Stecher, and Kumar 2021) . The scale bar represents the number of nucleotide substitutions per site.

4. Discussion

The differences in the physical and chemical characteristics of water samples taken from various sites in Jordan are believed to affect microbial distribution. In this study, water temperatures recorded on various sites ranged from 40°C to 55°C, and these conditions are known to support thermophilic bacteria. This is in accordance with previous reports on bacterial growth in hot springs (Radaideh et al., 2010; Mohammad et al., 2017; Obeidat and Al-Shomali, 2023). Previous studies showed that the concentration of each chemical at a certain location defines the type of microbes inhabiting that location (Fomina and Skorochod, 2020; Verma et al., 2022). Minerals are important components that affect the pH value of the surrounding environment and subsequently affect the growth of microorganisms (Hutchens, 2009; Fomina and Skorochod, 2020; Verma et al., 2022).

Ma'in hot springs and Zara Dead Sea water, containing high mineral content of sodium and chloride ions only, align with previous studies that identified Jordan hot springs and the Dead Sea saline environment as rich in salts that aid the growth of halophilic microbes (Hussein et al., 2017). This is further supported by (Jacob et al., 2017) reporting the same results on the microbial diversity of Dead Sea water and its surrounding regions.

The water sample obtained from North Shounah exhibited higher concentrations of magnesium and bicarbonate compared to samples from the Ma'in and Zara sites. This unique composition agrees with the previous data reported by Schäffer and Sass (2014). It is important to note that variations in mineral contents, like magnesium and bicarbonates, were found between water samples collected from different sources in Jordan. These variations may affect the growth of microorganisms in these water sources. The Mekhebh water has some different characteristics from an ecological point of view, such as sodium and chloride being lower than in the other areas, and bicarbonate being higher. As of now, there are no other studies that are focused directly on the Mekhebh hot springs.

Based on the phylogenetic analysis, it can be concluded that the bacteria isolated from the four hot springs are diverse and, in some ways, could adapt to the thermal conditions.

Moreover, among many strains of thermophiles isolated from various hot springs, *Anoxybacillus* was found to be the predominant genus (Ulucay et al., 2022) and (Ortega-Villar et al., 2024). This study found that MN1S1, MN6 S1, MN9 S1, and SH11 S3 isolates were closely related to *A.flavithermus* subsp. *flavithermus* strain DSM 2641; this strain is known as a thermophilic bacterium that lives under extreme temperature conditions. These findings agree with previous observations made about *A.flavithermus* in such geothermal environments, including those existing in similar areas but largely separated from Turkey (Agüloğlu Fincan et al., 2014), implying the occurrence of similar ecological niches provided by Jordanian hot springs. A clustering of several isolates (MN2 S1, MN3 S1, and MN4 S1) with the *T. taiwanensis* strain I1 was another remarkable observation. It is important to note that the data presented in this study is the first report on the occurrence of *T.taiwanensis*in in Jordan. According to Chen et al. (2006), this thermophilic, facultative anaerobe bacterium has been reported for the first time in hot springs in Taiwan. Its presence in Ma'in hot springs indicates the ecological resemblance that exists between this environment and East Asian hot springs, particularly in temperature and chemical composition, which may favor colonization and adaptation of *Tepidimonas* species. Moreover, the fact that *Flavobacterium thermophilum* has been isolated from multiple sites (MN5 S1, MN8 S1, ZARA1, ZARA3, SH3 S3, and SH8 S3) also shows the diversity among organisms found in these thermal waters. First isolated from the Italian geothermal area (Yoshizaki et al.,1971), it is characterized by thermophilicity as well as degrading complex organic matter. The identification of this species from both Zara and North Shounah hot springs supports the notion that these places provide proper conditions for thermophilic and heterotrophic bacteria vital for nutrient recycling in extreme environments like hot springs, where these bacteria can thrive **(Singh et al., 2019; Delete)** Kochhar et al., 2022 and EFSA Panel on Food Enzymes (FEZ) et al., 2025). It is worth noting that *Bacillus licheniformis* was found to be a dominant species in many hot spring samples. This species has been heavily investigated for its thermophilic attributes and ability to produce enzymes of industrial significance like proteases and amylases (Schallmey et al., 2004). Eleven strains closely related to *Bacillus licheniformis* strain DSM 13 have been isolated from different hot springs, indicating that this bacterium is comfortable with the different ecological conditions of geothermal springs prevailing in Jordan. Moreover, its adaptation to extremely high temperatures and mineral concentrations has been recorded worldwide (Makowski et al., 2021).

The identification of *Anoxybacillus kestanbolensis*, *Anoxybacillus rupiensis*, and *Anoxybacillus contaminans highlights* the supremacy of the *Anoxybacillus* genus in those hot springs. These thermophilic and halophilic microbes were previously identified in other geothermal habitats (Narsing Rao et al., 2018). The occurrence of these microorganisms in the Ma'in and North Shounah hot springs can be attributed to high mineral concentrations, particularly sodium and chloride, which favor their growth. The close relationships among isolates that were located in clusters with high bootstrap values (>95%) show how these thermophiles are strongly related to their ecological niche.

Finally, the isolation of *Geobacillus stearothermophilus* and *Pseudoxanthomonas taiwanensis* from Zara and North Shounah hot springs emphasized the microbial diversity in these ecosystems. It is widely known that *G. stearothermophilus* has industrial use, especially in the biotechnology industry, because of its thermostable enzymes (Gandhi et al., 2015). This suggests that Jordanian hot springs have a high potential for microbial communities that are useful in biotechnology.

Yin et al. (2024) emphasized the increasing importance of thermophilic glycoside hydrolases (GHs) derived from hot spring microorganisms, highlighting their exceptional stability and catalytic efficiency at high temperatures. Their editorial underscored how advanced bioinformatics tools, metagenomic sequencing, and molecular dynamics simulations have enabled the identification and functional validation of GHs with industrial significance. In

particular, enzymes such as β-glucosidases and xylanases exhibited resistance to inhibitors and retained functionality under extreme conditions. These findings complement the results of the current study, which identified thermophilic genera such as *Geobacillus* and *Anoxybacillus*, known for their production of thermostable hydrolytic enzymes. This supports the notion that Jordanian hot springs represent promising biotopes for the discovery of novel thermostable GHs with potential industrial applications.

Additionally, Guta et al. (2024) demonstrated the successful isolation of thermophilic strains from Ethiopian hot springs that produce amylase, protease, cellulase, and lipases, illustrating the functional diversity of hot springs microbiota under similar thermal conditions.

The discovery of thermophilic bacteria in Jordan's hot springs holds significant global industrial potential, particularly in biotechnology and bioengineering. Thermophilic bacteria, thriving in high-temperature environments, are invaluable for various industrial applications due to their unique enzymatic properties. For instance, they have been used for biofuel production, particularly in generating hydrogen through dark fermentation (Gallo et al., 2024). Thermophilic bacteria have also been studied for the bioremediation potential of municipal wastewater under high temperatures (Al-Rasheedi et al., 2022). Thermophilic bacteria have demonstrated significant potential in the biosynthesis of nanoparticles with diverse industrial applications. Recent studies have highlighted their role in producing nanoparticles with notable antibacterial properties. For instance, *Thermus thermophilus* has been shown to extracellularly synthesize silver nanoparticles (AgNPs) with potent antibacterial effects against both Gram-positive and Gram-negative bacteria (Romano et al., 2022).

The identification of *Bacillus licheniformis*, *Flavobacterium thermophilum*, and *T. taiwanensis* in this study further supports their relevance in industrial biotechnology. Previous research has shown that species such as *B. licheniformis* are prolific producers of thermostable proteases and amylases, enzymes highly sought after for use in food, textiles, and pharmaceutical industries (Ashaolu et al., 2025). The discovery of similar taxa in Jordanian hot springs strengthens the case for considering these sites as viable sources of thermostable enzymes.

The unique Thermophilic bacteria found in Jordan's hot springs could be crucial for developing new industrial products and processes, supporting advances in biotechnology and sustainable industry practices not only locally or regionally but also worldwide. These bacteria are specific to this region’s climate, abiotic factors, and conditions. This suggests that the metabolic and productive capabilities of these thermophiles could expand the global range of thermophilic industry products.

To conclude, the microbial diversity reported in Jordanian hot springs is indicative of how various environmental factors, such as temperature, salinity, and mineral content, contribute to microbial adaptation. The presence of diverse thermophilic genera like *Anoxybacillus*, *Tepidimonas*, *Flavobacterium*, and *Bacillus* shows that these hot springs provide a unique habitat for microorganism growth. The identification of *T. taiwanensis* for the first time in Jordan, together with other thermophiles clustering, suggests that there is much more to these hot springs than just being a reservoir of microbial diversity; they may also serve as an important ecological niche to study microorganism adaptation and evolution processes under extreme environmental conditions.

Furthermore, the presence of *Tepidimonas taiwanensis* in Ma’in hot spring, previously isolated only in Taiwan, suggests ecological parallels between distant geothermal environments and supports the idea that Jordanian hot springs could harbor microbial communities of global biotechnological importance (Zheng et al., 2025). These findings align with global trends where researchers seek extremophile enzymes due to their resilience under industrial stress conditions, including high temperature, salinity, and chemical solvents.

5. Conclusion

This study demonstrates that the physical and chemical parameter gradients of the Jordanian hot springs are a key factor influencing microbial populations. Elevated temperatures of the geothermal waters create an ideal habitat for thermophilic bacteria, and factors such as sodium, chloride, bicarbonate, and magnesium affect microbial distribution. Among the isolates, *Tepidimonas taiwanensis* and *Pseudoxanthomonas taiwanensis* were not previously recorded from Jordan, indicating an ecological similarity between the Ma'in hot springs and similar East Asian thermal ecosystems. The presence of *Anoxybacillus*, *Bacillus*, *Geobacillus*, and *Flavobacterium* also highlights microorganisms' ability to survive extreme conditions. The structure of this group of thermophile-associated bacterial isolates further suggests that these hot springs could serve as natural reservoirs for thermotolerant bacteria, with potential applications in biotechnology or industry.

Overall, the current study offers valuable data on the microbial diversity of Jordanian hot springs and their role as sources of thermophilic bacteria. Future research should explore the functional significance of these microbes, especially their enzyme capabilities and other biotechnological and medicinal applications.

Acknowledgments:

We gratefully acknowledge the Jordan Valley Authority for their valuable support in conducting the chemical analysis of water samples. We also wish to thank the University of Jordan for its financial support, which made this study possible.

Funding statement:

The Deanship of Scientific Research at the University of Jordan supported this research for graduate students [grant number 400].

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