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Diversity and Distribution of Thermophiles and Thermo-tolerant Bacteria in the Soil Samples Obtained from Different Regions in Saudi Arabia

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Soil bacteria play an essential function in different biogeochemical cycles. The present study aimed to estimate microbial diversity in five natural environments of Saudi Arabia by isolating and identifying thermophiles and thermo-tolerant bacteria. The collected soil samples were analyzed physically, chemically, and microbiologically. Biochemical and molecular techniques identified many bacteria, including *Enterobacter ludwigii, Enterobacter sp., Enterobacteriaceae bacterium, Bacillus sp., Bacillus subtilis, Bacillus licheniformis, Paenibacillus sp., Paenibacillusdendritiformis, Paenibacillus lactis, Pseudomonas aeruginosa, Pseudoalteromonas sp., Staphylococcus sp.,* and *Brevibacillus borstelensis.*This is the first report of the Enterobacter ludwigii isolate from the soil samples collected in Saudi Arabia to the best of the authors' knowledge, which is part of plant growth-promoting rhizobacteria (PGPR) can influence the composition of the rhizosphere soils, root tissues, and enhanced plant outgrowth. The presence of these bacteria could be utilized to promote agricultural practices in the deserts in Saudi Arabia.

Keywords: 16S rRNA, Thermophiles, Thermo-tolerant bacteria, Soil, Hot desert, Saudi Arabia.

Growth and adaptation of organisms in the hosting environment are affected by many factors, temperature in particular (Brooks *et al*.,2011). For organisms classified as thermo-tolerant, the mesophilic range (30"37 °C) is the most optimal; however, they are also able to grow in high-temperature environments in the philic range. On the other hand, the optimal growth temperature of organisms classified as thermophiles is much higher (60 °C), even though they were found to thrive in much hotter environments, such as terrestrial volcanic sites (Nazina *et al*., 2008). Microorganisms, specifically bacteria, are increasinglyfound to thrive in extreme conditions, such as those characterizing deserts, namely low nutrient status, extreme temperature fluctuations, high levels of UV radiation, and strong winds (Chamizo *et al*., 2012; Lester *et al*., 2007; Stomeo *et al*., 2013). Temperature is the dominant factor controlling the growth ofmicrobial species in the desert soil (Brooks *et al*., 2011). In the pertinent literature, a variety of thermophilic bacteria are

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described, which were extracted from various regions in theworld, including China (Lau *et al*., 2009), Turkey (Gul-Guven *et al*., 2008), Bulgaria (Derekova *et al*., 2008), India (Sharma *et al*., 2008), Greece (Sievert *et al*., 2000), Italy(Maugeri *et al*., 2001), Iceland (Takacs *et al*., 2001), and Saudi Arabia (SarhanandAlamrri 2014). Desert soils, irrespective of the locationfrom which they are obtained, typically comprise a number of universal phyla,including Proteobacteria, Bacteroidetes, and Actinobacteria (Chanal *et al*., 2006; Connon *et al*., 2007; Fierer *et al*., 2009; Lester *et al*., 2007).On theother hand, Cyanobacteria, Gemmatimonadetes, and Firmicutes (Bahl *et al*., 2011; Lacap *et al*., 2011; Makhalanyane *et al*., 2013; Richer *et al*., 2015) may be relatively more abundant in desert soils than in other biomes (Fierer *et al*., 2012). A wide range of molecular biology techniques can be employed in microorganism identification, such as 16S rRNA sequencing, rep-PCR profiling, and fatty acid methyl ester, which can be used in microorganism characterization at both species and subspecies levels (Adiguzel, 2006; Nazina *et al*., 2008; Zaliha *et al*., 2007). These techniques are also valuable for studying ecosystem diversity, in particular for analyzing the phylogenetic relation between strains, and discriminatingmicroorganisms that are genetically close to each other (Adiguzel, 2006).

The majority of research in microorganisms of the desert ecosystem was limited to few desert sites, particularly, in America and Australia. Consequently, significant effort is needed to expand to these studies to other regions, e.g., Asian and Africa. The more diverse data about microbial communities we have, the batter we are able to predict their impact on climate and land-use change (Makhalanyane *et al*., 2015). Subsequently, this study seeks to investigate microbial communities in hot deserts in Saudi Arabia.

The aim of this study was to identify and characterize thermophiles and thermo-tolerant bacteria isolated from soil in Saudi Arabia. In order to ensure diversity in soil samples, these were collected from Riyadh (central region), Dammam (eastern region), Hail (northern region), Abha (southern region), and AlmadinaAlmonawara (western region) via phenotypic and genotypic methods.

MATERIALS AND METHODS

Sample Collection

Soil samples were obtained from natural ecosystems by collecting specimens of 20–40 g wet weight from the top 50 cm layer aseptically, which wereplaced in sterile glass containers and boxes kept at about 4 ^{o}C during transport.

To ensure diversity, samples were collected from several sites, is illustrated in Figure 1,located in northern, southern, eastern, western, and central regions of Saudi Arabia.

Chemical Analysis of Soil

pH of the soils was determined by using an electronic pH meter (EckertandSims, 2011), and total salt concentration was determined by using Mehlich 3 method (Wolf and Beegle, 2011). Particle Size Analysis was conducted using Hydrometer Method (Gavlak,*et al*., 2005).The obtained results were presented as mg/l of dry soil.

Microbial Analysis

Each soil samplewas grown in 250ml flasks using 10g / 90ml of liquid media for enrichment,namely nutrient broth (v/v) for bacteria. The flasks were incubated at 45^{o}C for 6 days. The Bacteria in the samples was enriched and isolated using the solid medium. Inoculum with adequate turbidity was transferred to three agar media, namely blood, nutrient, and MacConkey agar for bacteria. Each bacterial sample was incubated at four different temperatures (45°C, 50°C, 55°C, and 60°C) for 48"72 h. For all species, purification was carried out by applying the streaking plate method. Bacterial colonies were identified using microscopic examination, morphological analyses, and a biochemical kit.

DNA Extraction and 16S Ribosomal RNA-PCR Analysis

Bacterial DNA isolates was taken from 5 ml bacterial cultures grown overnight using DNeasy Blood & Tissue Kit (Qiagen, cat. #69504) for DNA isolation. Samples were processed as per the instructions in the kit.DNA amplification reactions were carried in Veriti® Thermal Cycler (AB, Applied Biosystems). The small-subunit rRNA (16S rDNA) were amplified by primers targeted to universal regions. The primers had the following sequences: universal forward primer Bac27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and universal reverse primer Bac1492R (5'-

CGGTTACCTTGTTACGACTT-3'), used to amplify bacterial 16S rRNA. PCR amplifications were put according to the protocol described earlier (Flanagan *et al*., 2007).The PCR product was analyzed on 2.0% agarose gel with 0.5 ìg/ml ethidium bromide and was imaged using Bio-Rad Gel Documentation System 2000.

16S rRNA Sequence Analysis

The 16S rRNA gene of the isolates was sequenced using ABI 3700 DNA Analyzer (Applied Biosystems, USA).BLAST algorithm in Gen Bank was used for analyzed homology of the 16S rRNA gene sequence of the isolates based on the available reference 16S rRNA sequences. MEGA version 7.0 software (Kumar *et al*., 2016) was employed when conducting phylogenetic and molecular evolutionary analyses.

RESULTS

Physical and Chemical Characteristics of Soil from Different Regions

Five locations were prospected in Saudi Arabia, whereby the soil samples were obtained from Riyadh (central region), Dammam (eastern region), Hail (northern region), Abha (southern region) and AlmadinaAlmonawara (western region).The results of physical and chemical

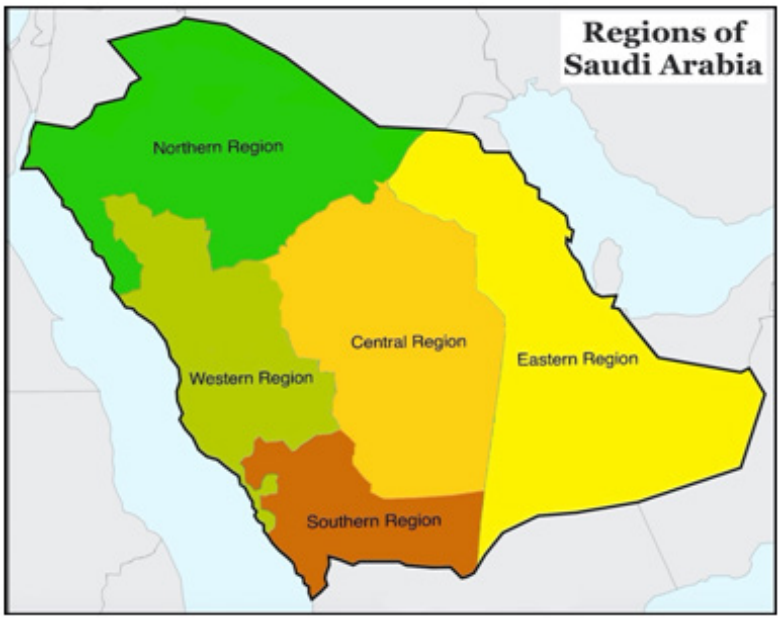

Fig. 1. Map of regions of Saudi Arabia

Table 1. Physiochemical Characteristics of Soil Samples

Characteristics	Soil Sample Location				
	East	Centre	North	South	West
Texture	L	LS	LS	LS	LiS
Clay %	16.8	6.8	4.3	6.8	24.3
Silt %	35	5	1.25	7.5	60
Sand %	48.2	88.2	94.45	85.7	15.7
pH	8.12	8.2	8.45	7.94	8.38
EC ms/cm	3.83	0.38	1.21	2.02	1.99
Na (ppm)	178	49	63	35	840
P (ppm)	8.4	7	9.8	30	8.4
K (ppm)	156	96	103	90	305
$CaCl_2$ %	10.2	2.29	2.9	2.6	13.03

Table 2. Nine Bacterial Genera Identified in the Soil Samples

Temp.	Location				
	East	Centre	North	South	West
	Clostridium sporogenes *Enterobacter ludwigii* *Enterobacter ludwigii* *Enterobacter ludwigii* *Brevibacillus borstelensis* *Paenibacillus* sp. *Enterobacter* sp.	*Enterobacter* sp. *Bacillus* sp.	*Paenibacillus dendritiformis* *Paenibacillus dendritiformis* *Paenibacillus dendritiformis*	*Paenibacillus*sp *Pseudomonas aeruginosa* *Enterobacter* sp.	*Paenibacillus lactis* *Paenibacillus lactis* *Paenibacillus lactis* *Paenibacillus*sp.
55 °C	*Paenibacillus*sp. *Enterobacter* sp. *Bacillus cereus* *Pseudomonas aeruginosa*	*Paenibacillus*sp. *Staphylococcus* sp. *Pseudomonas aeruginosa* *Enterobacter* sp.	*Bacillus subtilis* *Staphylococcus* sp.	*Enterobacter ludwigii* *Cedeceadavisae* *Pseudomonas aeruginosa*	*Bacillus subtilis* *Bacillus licheniformis*
50 °C	*Enterobacter ludwigii* *Bacillus licheniformis*	*Bacillus subtilis*	*Enterobacter ludwigii* *Enterobacter ludwigii*	*Enterobacter ludwigii* *Pseudoalteromonas*sp.	*Enterobacter ludwigii*
45 °C	*Enterobacter hormaechei* *Enterobacter ludwigii* *Enterobacter ludwigii* *Brevibacillus borstelensis*	*Enterobacter ludwigii* *Enterobacter ludwigii* *Bacillus subtilis subsp.*	*Enterobacter* sp.	*Escherichia* sp. *Enterobacter ludwigii* *Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* *Enterobacter* sp. *Bacillus* sp. *Enterobacter ludwigii*

analyses performed on the fivesoil types are summarized in Table 1. During September 2014, temperaturesin Saudi Arabia were in the 31"43 °C range.The analyses revealed that all soil samplescontained slightly alkaline water (pH = 7.9–8.5), as well as high potassium concentrations.In addition, soil samples collected from the Dammam and Almadina locations had high concentrations of sodium,phosphate,and calcium chloride.

Among the 57 isolates of thermophilic and thermo-tolerant bacteria that grew on different agar media at 45"60°C, 16(28.1%)were obtained fromthe eastern region, 11(19.3%) from the central region, 7 (12.2%) from the northern region, 11 (19.3%) from the southern region, and 12 (21.1%) from the western region.

Further analyses confirmed that nine bacterial genera were identified across the soil sampling sites, with *Enterobacter* genera being the most dominant, and*Clostridium* and *Cedecea*being the least prevalent, as indicated in Table 2. Other bacterial genera included *Pseudomonas, Staphylococcus, Bacillus, Paenibacillus,* and*Brevibacillus.* Moreover, three species were found to be thermophiles, *Clostridiumsporognes*, *Paenibacillusdendritiformis*, and *Paenibacillus lactis*, as they only grow under temperature condition of 60 °C. Whereas, all other isolates were found to be thermo-tolerant, as they grow under temperature conditions of 45-60 °C.

16S rRNA Sequence Analysis

Species level confirmations of 27 isolates were performed by 16S rRNA sequencing. Based on the findings yielded by the BLAST search analysis of the sequences, the isolates showed maximum identity (99%). The isolate sequences have been deposited in GenBank, as

Table 3. Identity of the 27 Thermophilic and Thermo-tolerant Bacterial Isolates Based on BLAST Searches

Code (accession number)	Identity Based on BLAST Searches	Max Identity (%)	GenBank Accession No.	E-value (Query Coverage %)
KF1 (MF682065)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF2 (MF682066)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF3 (MF682067)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF4 (MF682068)	*Enterobacter ludwigii*	97%	KM077046.1	0.0 (99)
KF5 (MF682069)	*Enterobacter* sp.	99%	KR856429.1	0.0 (100)
KF6 (MF682070)	*Enterobacter* sp.	99%	KC342873.1	0.0 (100)
KF7 (MF682071)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF8 (MF682072)	*Enterobacter hormaechei*	96%	KU312822.1	0.0 (100)
KF9 (MF682073)	*Enterobacter ludwigii*	96%	KM077046.1	0.0 (98)
KF10(MF682074)	*Enterobacter ludwigii*	98%	KM077046.1	0.0 (99)
KF11(MF682075)	*Enterobacter* sp.	99%	KR856429.1	0.0 (100)
KF12(MF682076)	*Bacillus* sp.	99%	HM566879.1	0.0 (95)
KF13(MF682077)	*Enterobacter ludwigii*	99%	KX024731.1	0.0 (100)
KF14(MF682078)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (99)
KF15 MF682079	*Enterobacter ludwigii*	98%	KM077046.1	0.0 (99)
KF17(MF682080)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF18(MF682081)	*Enterobacter* sp.	98%	KC342873.1	0.0 (100)
KF19(MF682082)	*Enterobacter* sp.	98%	MF125281.1	0.0 (99)
KF20 (MF682083)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF22 (MF682084)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF23 (MF682085)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF24 (MF682086)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF25 (MF682087)	*Paenibacillus* sp.	99%	KR364781.1	0.0 (96)
KF26 (MF682088)	*Enterobacter ludwigii*	99%	KM077046.1	0.0 (100)
KF27 (MF682089)	*Enterobacter* sp.	98%	MF125281.1	0.0 (100)
KF28 (MF682090)	*Bacillus* sp.	99%	KF217252.1	0.0 (100)
KF29 (MF682091)	*Enterobacter ludwigii*	98%	KM077046.1	0.0 (100)

follows:*Enterobacter ludwigii*with accession numbers MF682065, MF682066, MF682067, MF682068, MF682071, MF682073, MF682074, MF682077, MF682078, MF682079, MF682080, MF682083, MF682084, MF682085, MF682086, MF682088, and MF682091;*Enterobacter sp.* with accession numbers MF682069, MF682070, MF682075, MF682081, MF682082, and MF682089;*Enterobacter hormaechei*with the accession number MF682072;*Bacillus sp.* with accession numbers MF682076 and MF682090; and *Paenibacillus sp.* with the accession number MF682087 (Table 3).

The phylogenetic analyses of the 27 thermophilic and thermo-tolerantbacterial isolates and closely related species were conducted using the neighbor-joining tree method, as shown in Figure2. The generated dendrogram revealed two clades supported by high bootstrap values. These clades are represented by two major lineages, namely Proteobacteria (89%) consisting mainly of the genera*Enterobacter*, and Firmicutes consisting of the genus*Bacillus* (11%).

We found that the *Enterobacterludwigii*was the most dominant species of the identified genera *Enterobacter* (71%). The phylogenetic tree of the 17*Enterobacterludwigii*strains and the closest NCBI (BLASTn) strains—KM077046.1 and KX024731.1—based on the 16S rRNA gene sequences (neighbor-joining tree method) is

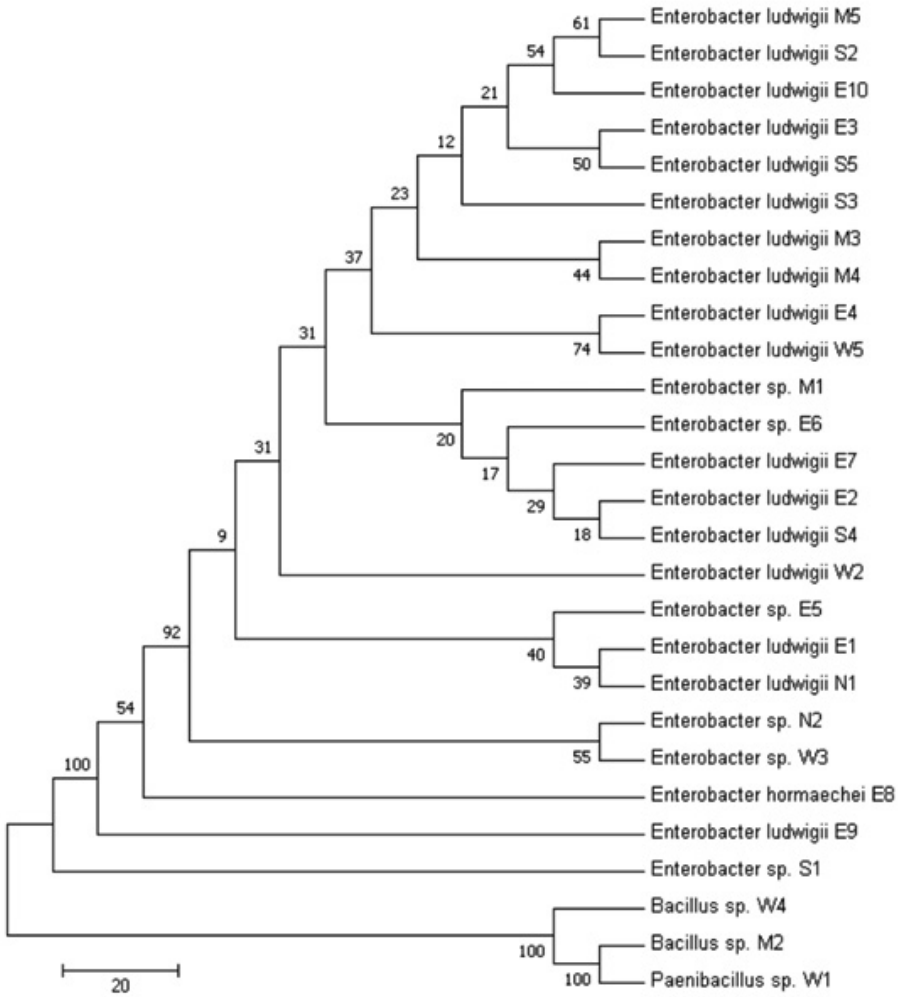

Fig. 2. Phylogenetic analysis of the two clades based on the 16S rRNA gene sequences and neighbor-joining tree method analysis results

illustrated in Figure3. A high similarity (~ 99%) with the reference strains available in the GenBank databases was identified (Table 3). Thirty-five percent of the 17 *Enterobacterludwigii* strains were found in the Eastern region of the country. To the best of the authors' knowledge, this is the first report of the *Enterobacterludwigii*isolate from the soil samples collected in Saudi Arabia.

DISCUSSION

In this study we have isolated and identified different bacteria that grow and survive at high temperatures from 5 five different regions in the Kingdom of Saudi Arabia. We identified four genera of thermophilic and thermo-tolerantbacteria isolated from soil samples.This study shows that Proteobacteria and Firmicutes were the dominant phyla in the microbiota of the Soil Samples. Interestingly, these two phyla were also found to be dominant in hot springs (Lee *et al*., 2018). Analyses also revealedthe presence of 57 thermophilic isolates pertaining to*Enterobacter, Bacillus, Paenibacillus,and Pseudomonas*.Other studies have identified *Bacillus* genus, specifically the *Bacillus licheniformis*, in hot springs, deserts and salt marshes in Morocco and in hot spring in Jorden (Aanniz *et al*., 2015; Mohammad *et al*., 2017; Al-Shammary *et al*., 2017;BahkaliandKhiyami, 2008; Khalil, 2011). In addition, a study in the Northern region of the Kingdom of Saudi Arabia, Hail, identified *Bacillus* and *Staphylococcus*in soil isolates similar to our findings of the northern soil samples (Al-Shammary *et al*., 2017). A nation-wide study on thermophilic organism soil isolates has also identified all the reported genus in this study (BahkaliandKhiyami, 2008). *Bacillus* and *Brevibacillus*genus were identified in hot springs in the Kingdom of Saudi Arabia (Khalil, 2011). In another investigation, Alotaibi *et al*. 2020 have proven a wide variety of microbial communities in various areas that varied in physiochemical soil features in Saudi Arabia. Also, they have proven that higher fungal diversity than bacterial was isolated from desert areas and Sabkha. Also,Murgia *et al*. 2019 have showed significant

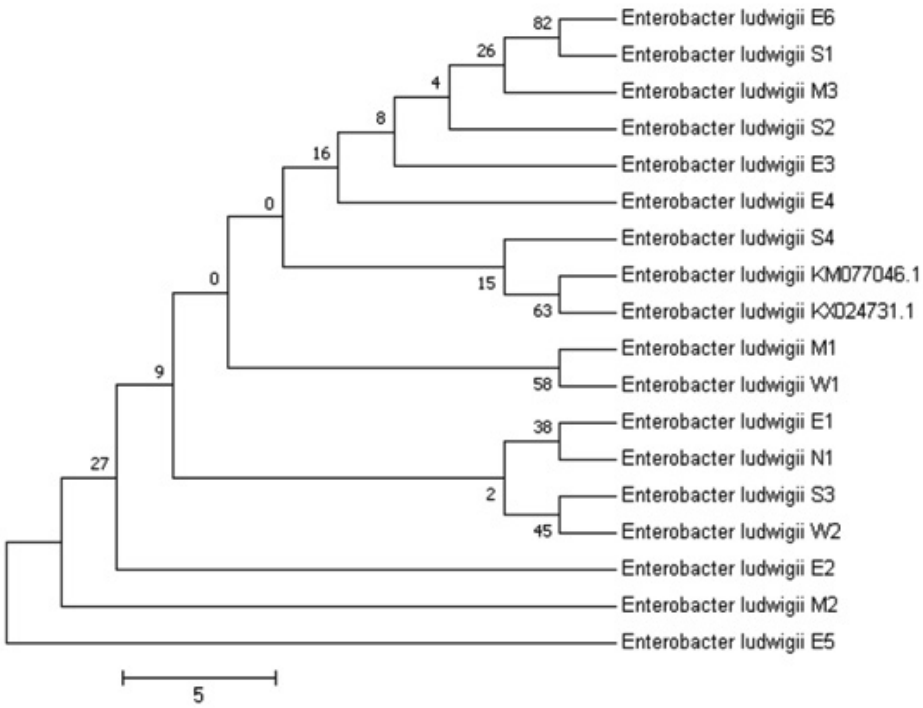

Fig. 3. Phylogenetic tree of the *Enterobacterludwigii* strain and the closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences (neighbor-joining tree method)

fungal biodiversity in the Middle East desert soil.

The highest and lowestpercentages of bacterial species wasnoted in the samples collected from in the eastern and northern region, respectively. On the other hand, we observed similar percentages were found in the central and southern regions of Saudi Arabia.*Enterobacter ludwigii* was the most common bacterial species, followed by *Enterobacter sp.* and *Bacillus*sp.To our knowledge, we are the first to identify this species of *Enterobacter* in the Kingdom of Saudi Arabia.

CONCLUSION

The results yielded by the present study indicate that numerous thermophilic and thermo-tolerant bacteria species thrive in different regions of Saudi Arabia.Moreover, although samples were collected from different regions to ensure diversity and comprehensive geographic coverage, the isolated bacteria species were generally similar. The abundance of bacteria in this study was typical of the environment with functional diversity and high species richness.Consequently, the findings of this study will provide invaluable information to microbial ecologists, as a diverse set of microbial communities of hot deserts in Saudi Arabia was identified. In addition, identification of thermophilic bacteria could be later used for biotechnological industry. In our future studies, the aim will be to determine the genetic variance among isolated thermophilic and thermo-tolerant bacteria and affected downstream proteins.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Data Availability

The sequencing data generated in this paper have been deposited in the GenBank repository with accession codes provided in Table 3.

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