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Different ecological, medical, and industrial important bacteria harboring the soil of Hail, Kingdom of Saudi Arabia

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ABSTRACT

This study aims at unraveling the bacterial biodiversity of Hail soil to establish a baseline study that contributes to harnessing these bacteria in applications that benefit human beings. We collected two groups of soil samples; one group of the models contained wheat roots, and the second group was free of roots. Bacteria were isolated from these soils, DNA was extracted, 16srRNA from different isolates was amplified and sequenced, and the phylogeny tree was analyzed. The taxonomic relationship indicated that the isolates obtained were belonging to Proteobacteria, Actinobacteria, and Firmicutes. The bacteria affiliated with Proteobacteria's phylum were Stenotrophomonas, Klebsiella, Azospirillum and Calidifontimicrobium. Firmicutes include Bacillus and Actinobacteria represented by Nocardioides. The genera Bacillus, Stenotrophomonas, Calidifontimicrobium, and Nocardioides were associated with wheat's rhizosphere, while the others live free in the soil. The study concluded that Hail soil is a pool of bacteria affiliated to different phyla; they share genetic traits, tolerate harsh environmental conditions that lead them to play different crucial roles in the environment, and may contribute to all aspects of human life harnessed adequately. More studies using housekeeping genes, "omics" approaches, and studies examining these isolates' ability to withstand extreme environmental conditions are recommended to view more insights about these bacteria.

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Introduction

Recently, the world witnessed dramatic climate change due to grassing, extensive use of fossils, industrial waste accumulation, synthetic pesticides and fertilizers, and overuse, among other anthropogenic activities. These activities lead to ecological contamination, lined with food insecurity, which threatens human life. To conserve human energy and maintain ecological peace, seeking scientific innovative sustainable approaches. The most sustainable, eco-friendly, and cost-effective approach is harnessing soil and plant-associated microbes in agriculture, industry, and bioremediation processes. This necessitates the availability of information through screening and characterization of soil and plant microbiomes, which were now known as a new platform for the green revolution (1). For proper screening and characterization of the microbes, phylogenetic studies became of increasing interest, which assists in assigning the identity of the isolates based on their 16SrDNA genes (2) and other gene sequences.

Some studies in Saudi Arabia recently were conducted to identify bacterial diversity in different areas, such as (3). In addition, (4) studied the microbial content of the industrial site in Hail city. However, according to our knowledge, no extensive studies were conducted to screen the microbial content of the agricultural soil of Hail and the plant's microbiomes, especially cereals, which are used as human food like wheat, one of the most important cereals in the world, which are used widely for human food and grown in different environments (5). Therefore, this study is designed to screen and unravel the bacterial biodiversity of Hail soil to establish a baseline study that contributes to harnessing these bacteria in applications that benefit human beings.

Materials and Methods

Study area

Ha'il is located in the northwestern region of the KSA (27° 31′ 0″ N, 41° 41′ 0″ E). This area is considered an agricultural and pastoral province characterized by rich water resources, fertile soil, and a temperate climate. This has resulted in agricultural development based on agricultural products, e.g., grains, dates, vegetables, forage crops, and fruit production. Recently, a large percentage of the Kingdom's wheat production came from Ha'il Province. The area to the North East of Ha'il, 60 km to 100 km away, predominantly consists of irrigated gardens. Various crops and fruit trees are currently cultivated in Ha'il, including barley, corn, vine trees, date palms, citruses, and other

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economic crops.

Isolation of bacteria

This study obtained bacterial isolates from free soil samples and soils containing wheat roots. Pure cultures of the isolates were obtained using Plate count agar, EMB agar, and Salmonella-Shigella agar media. In addition, Yeast Extract Mannitol Agar (YEMA) without and with Congo Red was used for Rhizobia enumeration. The media constituents are as follows:

Plate Count Agar (PCA)

This medium was used to determine the total bacterial count. It was obtained in a dehydrated form and composed of (g/L): Yeast extract, 9.0; Tryptone, 7.0; Dextrose, 5.0; Agar 9.0. The medium was prepared according to the manufacturer's instructions using 17.5 g/liter of distilled water. The medium was allowed to boil in the water bath until it was completely dissolved. The pH was adjusted to 7.0, the medium was sterilized in an autoclave at 121º C for 20 minutes.

Yeast Mannitol agar (YMA) with and without Congo Red

This media was used to cultivate, isolate, and enumerate soil microorganisms like *Rhizobium*. It is composed of (g/L): Yeast extract, 1.00; Mannitol, 10.00; Dipotassium phosphate, 0.50; Magnesium sulfate, 0.20; Sodium chloride, 0.10; Calcium carbonate, 1.00 and Agar, 15.00.

Eosin methylene blue (EMB) agar

Eosin methylene blue agar (EMB) is a selective and differential medium to isolate fecal coliforms. Eosin Y and methylene blue are pH indicator dyes that combine to form a dark purple precipitate at low pH; they also inhibit the growth of most Gram-positive organisms.

***Salmonella-Shigella* agar**

Salmonella-Shigella agar or SS agar is a selective and differential medium for the isolation of pathogenic enteric bacilli, particularly *Salmonella* and *Shigella*, from stool, food, and clinical material. It is composed of Pancreatic casein digestion (2.5g), beef extract (5g), Sodium thiosulphate (8.5g), bile salts (8.5g), Brilliant Green (0.00033g), agar (15,0g), Lactose (10.0g), Sodium citrate (8.5g), Ferric Ammonium Citrate (1.0 g), pH final (7.0).

DNA isolation, 16srRNA gene amplification, and sequencing

The genomic DNA of the isolates was extracted by Macrogen, Inc., Seoul, Korea. The extracted DNA was used as a template in a 30-µL reaction mixture using an EF-Taq DNA polymerase (SolGent, South Korea). Amplification of 16srRNA was done using forward primer 27F5`(AGAGTTTGATCMTGGCTCAG)3` and reverse primer 1492R5`(TACGGYTACCTTGTTACGACTT)3`. PCR reactions were prepared to make a total volume of 20 µl as follows: 2 µl of 10x Taq PCR buffer, 1.6 µl dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µl template DNA, 0.2 µl KOMA Taq polymerase, and 13.2 µl double distilled water. PCR conditions were set as follows: one cycle of initial denaturation at 95°C for 1 minute, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 2 minutes, extension at 68°C for 1.5 minutes, and one cycle of final extension at 68°C for 10 minutes. PCR products were purified and sequenced.

Sequencing was done using Big Dye Terminator Cycle sequencing kit v.3.1 (Applied Biosystems, USA). The primers used for sequencing were 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'. The Sequences were resolved on Applied BioSystems model 3730 XL automated DNA Sequencing System at Macrogen, Inc., Seoul, Korea. The G+C content was calculated using APE (A plasmid Editor) software version 8.5.2.0. Sequences were deposited in GenBank under accession numbers: OM149841, OM333165, OM327661, OM327747, OM333164, OM333165, OM333894, OM333243 and OM333894.

Data analysis

The sequences were aligned with the available nucleotide sequences database in the GenBank using the algorithm BLASTN of the National Center of Biotechnology Information (NCBI) (6). The isolates were identified according to the percentage of sequence similarity with the sequence in the GenBank. Phylogeny tree analyses were done online at www.phylogeny.fr (7-13).

Results and discussion

The bacterial enumeration results (Table 1) showed that the agricultural soil in Hail is rich in different bacteria; the approximate number of bacteria ranged between 106 to 109 (CFU/g). Rhizobia is a significant component of Hail soil among these bacteria, as shown in Table (1) below. It seems that the bacterial content of the agricultural area of Hail is higher than its industrial area, where they were counted at about 104 -107 cfu/g of soil (4). Rhizobia can be exploited in legume domestication programs in arid regions for sustainable soil reclamation and maintenance.

Table 1. The bacterial enumeration in the soil of Hail district**.**

Soil samples	TVC (CFU/g)	EMB agar count (CFU/g)	SS agar count (CFU/g)	YMA (CFU/g)	YMA-CR (CFU/g)
1	3×10^{9}	ND	ND	$8.2x10^{7}$	$2.5x10^{8}$
2	6.5×10^{6}	ND	ND	10^{7}	8×10^{6}
3	2.3×10^{8}	ND	ND	3.1×10^{8}	4.7×10^{8}
4	2.4×10^{7}	ND	ND	2.3×10^{7}	1.4×10^{8}
5	1.5×10^{8}	ND	ND	3.1×10^{9}	3.4×10^{8}
6	4.2×10^{8}	4.6×10^{2}	ND	2.5×10^{8}	5.4×10^{8}
7	1.5×10^{8}	9.3×10^{2}	ND	1.25×10^{8}	1.5×10^{8}

TVC: Total Viable Count on plate count agar (CFU/g); YMA: Total *Rhizobium* on Yeast Mannitol Agar (CFU/g); YMA-CR: Total *Rhizobium* on Yeast Mannitol Agar-Congo Red (CFU/g); EMB: Eosin methylene blue (EMB) agar count; SS agar: salmonella-shigella agar count.

The taxonomic relationship in this study showed that the isolates obtained belonged to Proteobacteria, Actinobacteria, and Firmicutes phyla. In the phylum Proteobacteria, the Gammaproteobacteria class was found to include the genera Stenotrophomonas and Klebsiella. Alphaproteobacteria class represented by Azospirillum and Calidifontimicrobium affiliated to the class Betaproteobacteria. The phylum Firmicutes includes species of the genus Bacillus and the phylum Actinobacteria represented by the genus Nocardioides. The sequence analysis of one isolate belonging to Gammaproteobacteria showed a similarity percentage of 99.42% to both Stenotrophomonas nitritireducens and Stenotrophomonas pictorum. Another isolate of the same genus was found similar to Stenotrophomonas pictorum by 99.08%. Stenotrophomonas species are known to play an important role in cycling elements in nature (14). For example, Stenotrophomonas nitritireducens reduce nitrite in nature without nitrogen gas production (2). No report was found on Stenotrophomonas nitritireducens pathogenesis in either humans or plants (15). In addition, Stenotrophomonas pictorum plays an important ecological role by degrading phenol, resorcinol, and 2- and 4-chlorophenol (16). However, Stenotrophomonas maltophilia is an opportunistic environmental bacterium and can infect humans and animals (17). Based on these findings, Stenotrophomonas obtained in this study are expected to play a role in the ecology and may be opportunistic or pathogenic bacteria.

One of the four isolates belonging to the genus Klebsiella was found similar to Klebsiella pasteurii, Klebsiella michiganensis, and Klebsiella oxytoca by 99.56%. The second isolate showed a similarity of 99.6% to Klebsiella oxytoca, and the last two isolates were found similar to Klebsiella aerogenes by 99.57 and 99.20%. The isolate belonging to Alphaproteobacteria showed low similarity to Azospirillum brasilense (82.33%). On the other hand, one of the two bacteria in the phylum Firmicutes showed a similarity of 99.85% to Bacillus haynesii, Bacillus glycinifermentans, Bacillus paralicheniformis, and Bacillus licheniformis; the second isolate was found similar to Bacillus paralicheniformis by 89.75%. The bacterium Bacillus haynesii is described as "a source of ZnO nanoparticles which are biologically active agents for testing human pathogens and could be considered for utilization in the green synthesis of nanoparticles for various medical and non-medical fields (18). Bacillus licheniformis produces 2, 3-Butanediol (19), which is used in pharmaceutical, food, cosmetic, energy industrial fields, used as a liquid fuel, plasticizer or antibiotic, and is also the starting material for the production of 1,3-butadiene, diacetyl, methyl ethyl ketone and polyurethane, which are the monomers or initial reagents for the synthesis of artificial gums, flavorings, and cosmetic products" (20). On the other hand, Bacillus glycinifermentans and Bacillus paralicheniformis were isolated before from Korean fermented "soybean paste food products". They were characterized by tolerance to high temperatures and high salt (21, 22).

In this study, the isolate affiliated with Actinobacteria was found 99.85% similar to Nocardioides terrigena, which is known live free in the soil (23). It can be useful for "bioremediation, biocatalysis, and metabolic engineering" due to its ability to cycle ethene (24). However, in this study, Nocardioides were associated with wheat's rhizosphere like the genera Bacillus, Stenotrophomonas and Calidifontimicrobium. In contrast, the rest isolates were found live free in the soil. This is partially consistent with previous fining reported that Stenotrophomonas and Bacillus were the most abundant in the wheat rhizosphere (5, 25). However, the bacteria that inhibit wheat rhizosphere may vary because "wheat genotype and physiological stage shaped the microbiome" (26). Even though in this study, Calidifontimicrobium was found associated with wheat, we did not find previous studies to support our findings; however, what we know is the free-living Calidifontimicrobium sediminis was isolated from hot springs in China (27).

Despite the sequence analysis results mentioned above, the phylogeny tree analysis placed heterogeneous bacteria in one-branched clad containing different bacterial groups (Figure 1). This clad contains Calidifontimicrobium, Bacillus, Stenotrophomonas, Klebsiella, and Azospirillum, although they belong to other classes. Some of these isolates are associated with wheat and some live freely in the soil. This finding can be interpreted by the possibility of acquiring or exchanging genes between these bacteria through horizontal gene transfer (28). This may be true for Nocardioides and Bacilli associated with wheat groups. Among the isolates obtained in this study, only four isolates were found placed in groups consistent with BLAST analysis. These include Stenotrophomonas KSA_H2 and Bacillus KSA_H2 living in the wheat rhizosphere and the free-living Klebsiella KSA_H1 and Klebsiella KSA_H2 shown in Figure (1).

As mentioned above and illustrated in Figure (1), Klebsiella KSA_H1 and Klebsiella KSA_H2 were found grouped with Klebsiella aerogenes, Klebsiella michiganensis, Klebsiella pasturii, and Klebsiella oxytoca. The isolates obtained in this study were found living free in the soil; however, in previous studies, Klebsiella michiganensis was isolated from a household toothbrush holder and characterized by salt tolerance (29). In addition, Podschun et al. (30) isolated Klebsiella oxytoca from freshwaters, which found less virulence. At the same time, Klebsiella oxytoca was found to infect humans and responsible for nosocomial infections (31). Klebsiella aerogenes was found resistant to multiple antibiotics (32). In addition, these bacteria were reported to produce 2, 3-Butanediol, a

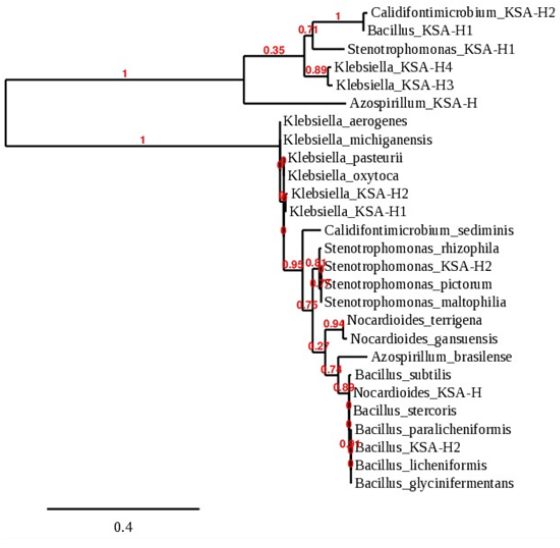

Figure 1. Phylogeny analysis of the different isolates obtained from wheat rhizosphere and soils of Hail.

compound whose derivatives are widely used in different vital industries (33), which is also produced by Bacillus licheniformis. These findings showed that our isolates in this study expected to have the ability to live in a different environment with multiple roles and may be of medical and industrial importance.

Finally, as stated in the sequences analysis section of this study, Azospirillum isolated showed low similarity to Azospirillum brasilense. Therefore, it is found the group with unrelated bacterial groups in the phylogeny tree. In agriculture, Azospirillum spp. can fix atmospheric nitrogen (34), and Azospirillum brasilense is reported to play a role in promoting plant growth (35), including forage plants (36), maize (37), rice and wheat (35).

It is a worthy note that the isolates in this study were characterized by low GC% compared to the isolates obtained in the previous studies. For example, the GC content of Stenotrophomonas and Nocardioides was found at 55%, while, for example, it was reported that the GC content of Stenotrophomonas pictorum is 65.7% (38) and Nocardioides terrigena 71.5% (23). Nevertheless, Klebsiella isolates in this study were characterized by high GC contents, while Klebsiella pasteurii to which our isolates related, was found of 54.6% GC content (29). The importance of soil has been described in many agricultural researches (39-44).

Hail soil is a pool of bacteria affiliated with Proteobacteria, Actinobacteria, and Firmicutes. These bacteria share genetic traits, which may be via horizontal gene transfer, they share genetic traits and may tolerate extreme environmental conditions, which make them the ability to play different crucial roles in all aspects of human life, such as material cycling in nature, plant growth promotion, sources of raw materials for different industries. Some may be of medical importance, like those living free in the soil. The assumption that horizontal gene transfer occurred between these heterogeneous groups of bacteria necessitates using housekeeping genes to deep study the taxonomic relationship between these bacteria. In addition, studies using approaches like genomics, transcriptomics, proteomics, metabolomics….etc may give more insights into these populations of bacteria. Furthermore, studies on the ability of these isolates to tolerate extreme environmental conditions recommend.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

A. E. Sulieman, A. Idris, S. Kahrizi– developed the concept, designed the experiment and wrote the manuscript; N. Alanaizy, N. Alshammari collected data and performed analyses; A. E. Sulieman, A. Alshammari, N.

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