

High-Throughput Sequencing-Based Review of *Bacillus* Diversity in Jordanian Hot Springs Water

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Abstract: *Bacillus* or *Bacillus*-related genera were the most frequently documented isolates from Jordanian hot springs in several earlier studies based on culture-dependent methods. This study aimed to review the abundance and composition of the genus *Bacillus* in Jordanian hot springs (Ma'in and Afra springs) using a culture-independent approach by Illumina MiSeq. The studied samples included three samples from Ma'in hot springs (HS1, HS2 and HS3) and one sample from Afra hot springs (HS4). Relative to the domain *Bacteria*, the abundance of *Bacillus* was found to be very low: 2% (in HS1), 3% (in HS2), 0.5% (in HS3) and 1% (in HS4). However, the abundance in lower taxonomic ranks was found to be higher: at the level of phylum *Firmicutes*, the genus constitutes 7% in HS1, 41% in HS2, 31% in HS3 and 33% in HS4. *Bacillus* was found to dominate the order *Bacillales* and class *Bacilli* only in case of HS2 (64%). In case of HS1, HS3 and HS4, the abundance was 18, 47 and 39%, respectively. Finally, *Bacillus* was found to be dominant as compared to other genera within the family *Bacillaceae* in case of HS2 (84%), HS3 (77%) and HS4 (61%) but not in HS1 (22%) which was dominated by the genus *Anoxybacillus* (77%). In respect to genus composition, in all studied hot springs, the species *B. firmus* was found the dominant species. Nevertheless, other species with lesser abundances were also detected like *B. cereus*, *B. beringensis*, *B. weihenstephanensis*, *B. funiculus* and *B. Oceanisediminis*.

Key words: *Bacillus* • Jordanian Hot Springs • Illumina MiSeq

INTRODUCTION

Jordanian hot springs have attracted researchers over the last decades to explore their microbial composition [1-3]. Despite the abundance of hot springs in Jordan, most microbiological investigations have focused on Ma'in Hot Springs (37-63°C) located in the central part of the country [1-8] and Afra hot springs (44-48°C) located in the southern part of the country [2, 3&7]. Pioneering studies on Jordanian hot springs, especially Ma'in and Afra hot springs, were conducted for several

related purposes like enumeration of bacteria [1], isolation and identification of bacteria [4-6], or exploring the biological activities and potential biotechnological applications of the native bacteria [8, 9].

Most, if not all, of the aforementioned studies have utilized a culture-dependent approach to study the diversity and the inherent activity of the native thermophilic/thermotolerant bacteria in Jordanian hot springs. Accordingly, a large number of bacterial isolates were obtained and most of them were affiliated in the genus *Bacillus* [1, 4-8]. In some cases, where *Bacillus* is

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not the main genus to be described, genera related to *Bacillus* within the family *Bacillaceae*, such as *Geobacillus* and *Anoxybacillus*, were described as the main bacterial genera [2].

Similar studies in other parts of the world have revealed almost the same conclusion, i.e., *Bacillus* and *Bacillus*-related genera are the main genera in hot springs. For instance, a total of 79 bacterial isolates dominated by the genus *Bacillus* were obtained from four hot springs in Morocco [10]. In another study, a collection of 161 strains of thermophilic *Bacillus* were also isolated from Tunisian hot springs [11]. Additionally, different Indian hot springs were studied and several strains belonging to the genera *Bacillus* and *Geobacillus* were isolated [12, 13].

Recently, we have applied a culture-independent approach to reveal the microbial diversity in Jordanian hot springs represented by Ma'in and Afra hot springs [14]. As a spinoff of this study, important data about the diversity of certain genera became available. In this current study, we aimed primarily to review the abundance of the genus *Bacillus* in Jordanian hot springs (Mainly Ma'in and Afra springs) based on these data generated by the Illumina MiSeq which was not utilized before for this purpose. Secondly, the species composition of the genus *Bacillus* would be determined because most studies did not identify the thermophilic/thermotolerant *Bacillus* isolates to the species level.

MATERIALS AND METHODS

The new findings in this study were generated from unpublished metagenomic data emerged from a recent published work done by our group on samples obtained from Ma'in and Afra hot springs [14].

Sampling of Hot Springs Water: Water samples were collected from four Jordanian hot springs; three samples were collected from Ma'in hot springs (HS1, HS2 and HS3) and one sample from Afra hot springs (HS4) as mentioned before [14]. Sample collection was carried out in June, 2015 as mentioned previously by our group and the *in situ* temperature of water ranged between 38 to 59°C whereas the pH values ranged between 7.4 to 8.4 [14].

DNA Isolation: DNA was isolated as described before [14], briefly, water samples were filtered under vacuum using 0.2 μm - nitrocellulose membranes. The nitrocellulose membranes containing the trapped cells were cut into pieces and transferred to sterile 50 mL centrifuge tube. The E.Z.N.A[®] Water DNA kit (Omega

Biotech Ltd., India; catalog No. D5525-01) was used for DNA extraction. The resulted DNA was finally stored at -20°C for further analysis.

Amplicon Sequencing: Amplicon sequencing using next generation technology (bTEFAP[®]) was used to analyze the water samples as described previously [14]. Briefly, the 16S universal eubacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to evaluate the species richness and abundance of each sample including the genus *Bacillus* on the Illumina MiSeq with methods via the bTEFAP[®] DNA analysis service. The PCR using Hot Star Taq plus Master Mix Kit (Qiagen, Valencia, CA) was done as described previously [14]. Samples were sequenced utilizing the Illumina MiSeq chemistry following manufacturer's protocols. The Q25 sequence data derived from the sequencing process were processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX).

Data Analysis: Visualization and analysis of results was done using Krona software. Krona software allows hierarchical data to be explored with zooming, multi-layered pie charts. Microsoft Excel 2010 was also used to generate the abundance charts.

RESULTS

Abundance of the Genus *Bacillus*: The abundance of the genus *Bacillus* within the family *Bacillaceae* was determined relative to different taxonomic levels. The genus *Bacillus* was found predominating the family *Bacillaceae* in three samples out of four (HS2, 84%; HS3, 77% and HS4, 61%; Table 1). However, in sample HS1, the genus *Bacillus* was found to be the second most abundant genus (22%; Table 1). The abundance of *Bacillus* was also calculated relative to higher taxa. The genus was dominating at the order *Bacillales* and class *Bacilli* only in case of HS2 (64%). However, the abundance was lower in HS1 (18%), HS3 (47%) and HS4 (39%; Table 1). At the level of the phylum *Firmicutes*, the genus constituted 7% in HS1, 41% in HS2, 31% in HS3 and 33% in HS4 (Table 1). Relative to the domain of *Bacteria*, the abundance of the genus was found to be very low: 2% (in HS1), 3% (in HS2), 0.5% (in HS3) and 1% (in HS4; Table 1). Because *Bacillus* was not the dominant genus within the family *Bacillaceae* in HS1, further investigation was carried out to clarify the relative abundance of genera other than *Bacillus* within the family

Table 1: Abundance percentage (%) of the genus *Bacillus* relative to each taxonomic rank in the water of four Jordanian hot springs HS1, HS2, HS3 and HS4

| | HS1 | HS2 | HS3 | HS4 |
|--|-----|-----|-----|-----|
| Genus (<i>Bacillus</i>): Domain (<i>Bacteria</i>) | 2* | 3 | 0.5 | 1 |
| Genus (<i>Bacillus</i>): Phylum (<i>Firmicutes</i>) | 7 | 41 | 31 | 33 |
| Genus (<i>Bacillus</i>): Order (<i>Bacilli</i>) | 18 | 64 | 47 | 39 |
| Genus (<i>Bacillus</i>): Class (<i>Bacillales</i>) | 18 | 64 | 47 | 39 |
| Genus (<i>Bacillus</i>): Family (<i>Bacillaceae</i>) | 22 | 76 | 61 | 84 |

*Numbers express a percentage

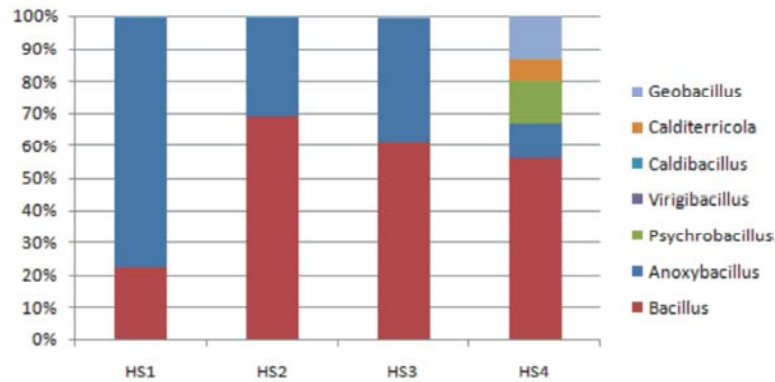


Fig. 1: The major genera within the family *Bacillaceae* and their relative abundance in Ma'in and Afra hot springs. The genus *Bacillus* is the dominant in HS2, HS3 and HS4 but not in HS1, where the genus *Anoxybacillus* dominates

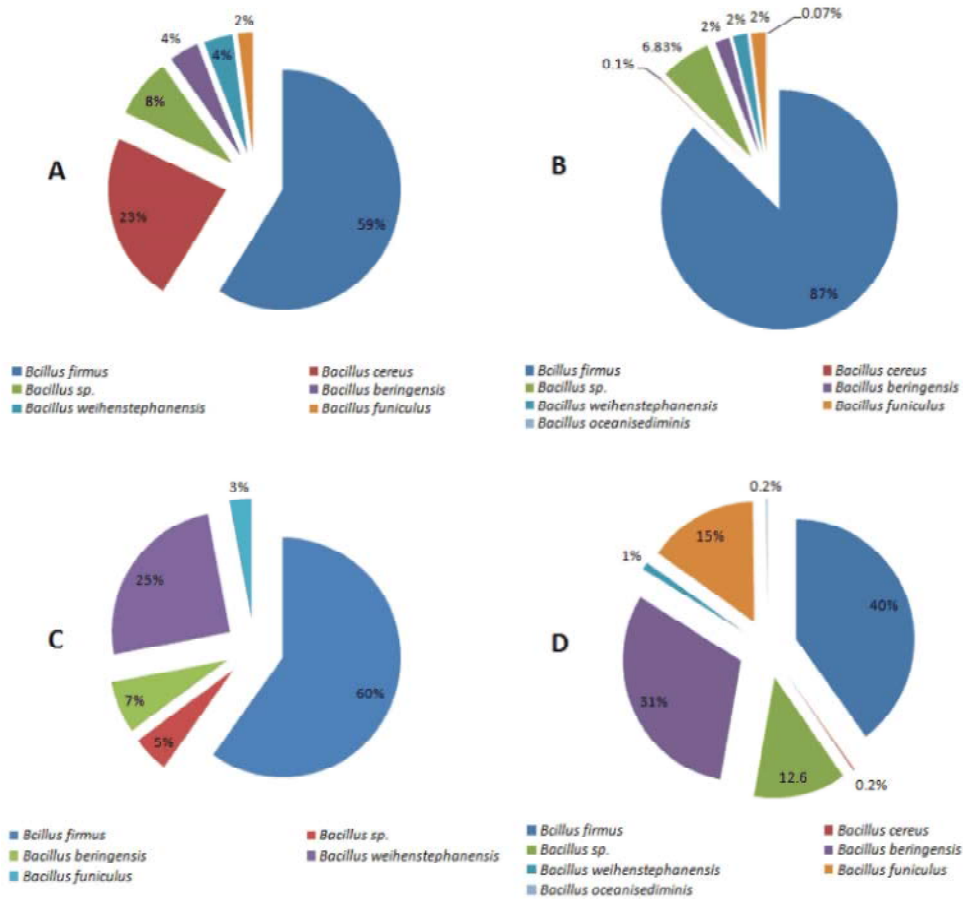


Fig. 2: Species diversity within the genus *Bacillus* in Jordanian hot springs: HS1 (A), HS2 (B), HS3 (C) and HS4 (D)

Bacillaceae. *Bacillus* was the second most abundant genus after *Anoxybacillus* (77%) in HS1 indicating that *Anoxybacillus* is the dominant genus in this sample. Generally, it can be concluded that the most abundant genera within the family *Bacillaceae* were *Bacillus* and *Anoxybacillus* in the four hot springs. The other genera related to *Bacillus* and *Anoxybacillus* within the family *Bacillaceae* that were detected but with lower abundance included *Psychrobacillus*, *Virigibacillus*, *Caldibacillus*, *Claditerricola* and *Geobacillus*. Fig.1 shows the major genera within the family *Bacillaceae* and their relative abundance in Ma'in and Afra hot springs.

Species Diversity Within the Genus *Bacillus*: The species diversity within the genus *Bacillus* was also determined for the studied samples. In all studied hot springs, the species *Bacillus firmus* was the dominant species. Additionally, there were other species of *Bacillus* detected in each sample. In detail, there were 6 species detected in the genus *Bacillus* in sample HS1 and their relative abundance was determined. As mentioned previously, the genus *Bacillus* in sample HS1 was dominated by the species *Bacillus firmus* (59%), followed by *Bacillus cereus* (23%), *Bacillus beringensis* (4%), *Bacillus weihenstephanensis* (4%), *Bacillus funiculus* (2%) (Fig. 2A).

In sample HS2, there were 7 species detected in the genus *Bacillus*. The most abundant species was again *Bacillus firmus* (87%), followed by: *Bacillus beringensis* (2%), *Bacillus weihenstephanensis* (2%), *Bacillus funiculus* (2%), *Bacillus cereus* (0.1%), *Bacillus oceanisediminis* (0.07%) and *Bacillus* sp. (6.83%) (Fig. 2B). Similar results were also obtained from sample HS3 where 5 species in the genus *Bacillus* were detected. The most abundant species was also *Bacillus firmus* (60%), followed by *Bacillus weihenstephanensis* (25%), *Bacillus beringensis* (7%), *Bacillus funiculus* (2%) and *Bacillus* sp. (7%) (Fig. 2C).

In sample HS4, there were 7 species detected in the genus *Bacillus*. The most abundant species was also *Bacillus firmus* (40%), followed by *Bacillus beringensis* (31%), *Bacillus funiculus* (15%), *Bacillus weihenstephanensis* (1%), *Bacillus cereus* (0.2%), *Bacillus oceanisedimentis* (0.2%) and *Bacillus* sp. (12.6%) (Fig. 2D).

DISCUSSION

Surveying the literature that describes the microbiology of Jordanian hot springs indicate that most studies were done using culture-dependent methods.

Methods based on cultivation are important when microbial material is required; however, the major drawback of cultivation-based methods is that they may underestimate the species richness of the studied samples [15]. Even though cultivation was frequently used to assess diversity and community structure in certain habitats, several reports indicated that culture conditions that have been used to perform diversity studies usually recover on the order of 1% or less of the total number of organisms [16]. Studies based on 16S rRNA gene have already proved that microbial diversity was much broader than suggested by culture-dependent techniques [17]. Therefore, the diversity of microbial communities requires comprehensive reliable techniques like high-throughput sequence analysis. High-throughput sequence analysis was developed to generate large quantities of sequences from environmental samples for diversity analysis [16]. Such analysis of total environmental DNA produces a vast amount of information and provides a more complete representation of the microbial communities present in certain habitats including diverse and cryptic thermal environments [17].

In this study, high-throughput sequence analysis was applied to focus on the diversity of the genus *Bacillus* in selected Jordanian hot springs (Ma'in and Afra hot springs). The mentioned springs were selected because several microbiological studies were carried out to explore their microbial communities. These studies have concluded that *Bacillus* and/or *Bacillus*-related genera are the main genera inhabiting in the Jordanian hot springs [1-6]. In more details, one early study by Khalil *et al.* [1] showed that *Bacillus* spp. were dominant over the detected Gram-positive and Gram-negative bacteria in the thermal springs located along the Jordan Rift Valley. In another study, a new strain in the genus *Bacillus* was also described in Ma'in hot springs by Elnasser *et al.* [6]. Moreover, Malkawi and Al-Omari [7] reported that 97% of the recovered thermophilic isolates from different Jordanian hot springs belong to the genus *Bacillus*. In more recent study, Fandi *et al.* [8] isolated new thermophilic strains of the genus *Bacillus* from Ma'in hot springs and screened them for antimicrobial activity. Similar results regarding the dominance of *Bacillus* in hot springs water samples were obtained from a number of international studies [10-13, 18].

In this study, we evaluated the abundance of the genus *Bacillus* using a culture-independent method. As expected, the abundance of the genus relative to all members in the domain *Bacteria*, the phylum *Firmicutes* and the order *Bacillales*/class *Bacilli* was found to be very low.

Table 2: Types of media used in selected previous studies that have isolated *Bacillus* and *Bacillus*-related species from Jordanian hot springs

| Type of Media | Growth Temperature | Number of Isolates | Reference |
|---------------------------------|--------------------|--|-----------|
| Thermus media (ATCC medium 697) | 55°C | 23 isolates (Most of them are <i>Bacillus</i> species) | [1, 4] |
| Different modified media | 55°C | 5 isolates | [8] |
| Thermus medium | | | |
| Nutrient Agar | | | |
| Trypticase Soy Agar | | | |
| Castenholz TYE medium | | | |
| Halophile medium | >50°C | 128 isolates | [7] |
| Nutrient Agar | >50°C | 4 isolates | [2] |

Unsurprisingly, it can be concluded that the frequent isolation of *Bacillus* and *Bacillus*-related genera at the expense of other genera is indicative of culture bias and not representative of the prokaryotic diversity of Jordanian hot springs as commonly known. This can be attributed to several factors like the strategy of isolation/cultivation and the type of utilized media. A closer look at the media used for cultivation of thermophilic bacteria from Jordanian hot springs in selected previous studies is presented in Table 2.

It seems that most studies done on Jordanian hot springs have utilized media that favor the growth of *Bacillus* but not the other dominant bacterial species. One can argue that the utilized media are general purpose media and can be used to cultivate wide range of bacteria in the lab. However, it must be noted that recent studies have shown that some bacteria belonging to rarely cultured groups are able to grow on common media once they are pure but they are unable to grow directly from the environmental samples [19]. Thus, it seems that the media and the set of growth conditions were supportive for the growth of only a subset of the total microbial community present in Jordanian hot springs samples (i.e., the genus *Bacillus*); therefore, the other bacterial populations were not obtained. Even though the general concept presented here is not new and very well known [18-20]; the data accessible in this study gives new insights into the diversity of the genus *Bacillus* in Jordanian hot springs.

CONCLUSIONS

In conclusion, even though *Bacillus* and *Anoxybacillus* are the dominant genera within the family *Bacillaceae* in Jordanian hot springs (Ma'in and Afra hot springs), their abundance of relative to all members in the domain *Bacteria* is very low. Moreover, *B. firmus* represents generally the most abundant *Bacillus* species

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