Seasonal diversity of the bacterial communities associated with petroglyphs sites from the Negev Desert, Israel

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Abstract

Rock art sites in the form of petroglyphs are found in the Negev Desert in southern Israel. These petroglyph sites include thousands of images depicting the life and culture of people in the region over the years. As they are part of the desert environment, these petroglyphs are exposed to natural and anthropogenic weathering processes. In natural rock sites, it is known that climatic conditions influence weathering and biofilm community development (Friedmann in *Orig Life* 10:223–235, 1980; Li et al. in *PloS one* 11:(9),163–287, 2016) Therefore, in order to develop tools for preservation and remediation, it is imperative to ascertain possible seasonal diversity of surface-inhabiting bacterial communities. To do so, we undertook to study and characterize the microbial communities inhabiting the rock sites during winter and summer. Next-generation sequencing (NGS) technologies using 16S rRNA gene were implemented on rock-surface slab samples to identify the microbial communities. 16S rRNA gene sequencing data revealed the presence of five main bacterial phyla, among them Actinobacteria, Cyanobacteria, Proteobacteria, Bacteroidetes, and Chloroflexi across the analyzed sites. The predominant phylum was Cyanobacteria (10–60% relative abundance), followed by Actinobacteria (20–60% relative abundance), Proteobacteria (5–35%), Bacteroidetes (0.02–35%), and Chloroflexi (1–12%). There was no significant difference in the bacterial diversity between samples from winter and summer. Five main phyla were observed in samples from the two seasons, winter (December) 2017 and summer (July) 2018. This study provides an important first step in understanding the possible seasonal dynamic of bacterial communities associated with the petroglyphs.

Keywords Petroglyphs · Bacteria · Cyanobacteria · Biofilm · Negev Desert

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Introduction

The Negev Desert, situated in southern Israel, extends from the Sinai Desert region in the west to the Jordanian Arabian Desert in the east, covering approximately 13,000 km² (Fig. 1). The Negev Desert contains various cultural heritage landscapes and archeological remains, and among them, hundreds of petroglyphs sites. These sites, can be found spread throughout the Negev central highlands, and are considered as a unique cultural landscape. The petroglyphs are carved on the local Eocene and late Cretaceous limestone rocks (Evenari et al. 1982) that are covered with thin dark coatings (Fig. 1). The petroglyph sites include thousands of figurative images, geometric shapes, symbols, and inscriptions. The use of archeological dating techniques revealed that these engravings are from around 3000 BCE. Recently, as more sites are being revealed, there has been an increasing awareness of their importance as a valuable cultural heritage resource (Eisenberg-Degen 2012). Unfortunately, despite the increase in the public

Fig. 1 **a** Location of the Negev Desert in the south part of Israel. **b** Har Michya Petroglyph site in the Negev highlands arid zone. **c** View of the rock art site at Har Michya (30° 49' N, 34° 43' E). **d** Rock engraving: the hunter, Har Michya

interest, there is an increase in physical damage caused by vandalism to the petroglyph sites. In addition, since they are part of the natural Negev Desert environment, the petroglyphs are exposed to natural weathering processes. Physical weathering processes such as diurnal heating and cooling of rock surfaces, chemical weathering processes, and weathering induced by biological agents such as plants, lichens, and bacteria may all affect their preservation state. Various groups of bacteria and other microorganisms inhabit rocks and stones in desert environments. Despite the inhospitable conditions of extreme deserts, several microhabitats have been established that are most suitable for life. One such microhabitat consists of the pores and fissures inside rocks (endolithic growth) and the rock underside (hypolithic growth) where humidity may be maintained. These endolithic habitats may offer microorganism better moisture conditions and shelter from harmful environmental factors (Golubic et al. 1981; Gorbushina 2007; Wierchos et al. 2012). In general, microbial colonization is initiated by various phototrophic microorganisms (e.g., cyanobacteria, algae), which support further colonization by various heterotrophic and chemoautotrophic as bacteria and fungi. A vast number of studies revealed their composition diversity and structure under different environmental conditions (Danin 1999; Thulani et al. 2015; Gorbushina 2007; Smith et al. 2014; Villa et al. 2016; Chimienti et al. 2016; Li et al. 2016). Regarding hot desert environments, several studies have indicated that the endolithic communities comprise relatively simple communities dominated by cyanobacteria, with some heterotrophic components. It was found that in such communities, Archaea and Eukaryotes may be absent or present in low abundance (Büdel et al. 2004; Thulani et al. 2015; Wierchos et al. 2012). Despite the vast number of publications regarding microbial communities inhabiting rock and stone monuments, to date, research focusing on

interactions between climatic factors and bacterial communities specific to the petroglyphs sites in arid environment is still sparse. We, therefore, decided to investigate the rock microbial diversity during two seasons: the relatively wet winter (2017) and the dry summer (2018). Results from this study will provide insights into the diversity and complexity of the rock microbial communities and will likely contribute to the development of basic tools for preservation management of the rock art sites in the Negev Desert.

Materials and methods

Study site

The study site, Har Michya petroglyph park, is located in the central highlands of the Negev Desert (Fig. 1). Har Michya is situated 2–3 km west of the Byzantine town of Avdat in the central Negev (30° 49' N, 34° 43' E). This area is regarded as an arid desert zone (Fig. 1) and receives an average of 80–100 mm of rainfall annually mostly during the winter season. This is followed by a 3- to 5-month dry summer period (Hillel 1982) (Table S1). There are about 200 nights of dew/year providing abundant sources of liquid water for microbial lithobiontic colonization in this desert (Kidron 1999). All rock art found within this area, 965 panels with 5077 elements, have been previously documented (Eisenberg-Degen 2012).

Sampling

Sampling was carried out during winter (December) 2017 and summer (July) 2018 from identical rock types that were found adjacent to the petroglyphs at a distance of about 1 m from them (Fig. 2). Small slabs of weathered rock surface 2 cm ×

Fig. 2 Rock surface sampling, petroglyphs park, Har Michya, Negev Desert, Israel

2 cm in size were collected aseptically using a small sterile chisel. All the samples were obtained from the rock surface containing the dark coating layer as well as the bright bedrock. The stone samples were each placed in separate sterile test tubes (Fig. 2). During winter 2017, the group of samples included samples from opposite faces of the rocks. These were marked as samples C1.1, C1.2, C2.1, C2.2, C3.1, C3.2, C7.1, and C7.2. Four more samples were collected randomly and were marked as C4, C5, C6, and C8. Sample C6 was collected from a nearby rock that lacks macroscopically visible dark coating. In the summer (July) of 2018, four more samples were collected randomly from rocks at the same site by a similar procedure as described previously and were marked as 112, 122, 132, and 142. The samples were transferred to the laboratory in a cooler box and were stored at -20°C until processing.

Culture independent study: 16S rRNA gene sequencing

Total genomic DNA from the environmental samples was extracted from 0.5 g of crashed stone samples using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the protocol supplied by the manufacturer. The extracted DNA was submitted to the DNA Services Facility (DNAS), at the Research Resources Center, at the University of Illinois at Chicago (UIC) for bacterial small subunit (16S) ribosomal RNA (rRNA) gene sequencing using the Illumina MiSeq platform. Before sequencing, two steps of PCR amplification were performed. During the first PCR, fragments of the V3–V4 regions of the bacterial 16S rRNA gene were amplified using 341F/806R universal primers attached with 5' linker sequences, CS1, and CS2 (known as common sequence 1 and 2).

The primer-linkers sequences were 5'-ACAC TGACGACATGGTTCTACACTACGGGAGGCAGC AGTGGG (CS1_341F; forward primer) and 5'-TACG G T A G C A G A G A C T T G G T C T G G A C TACHVHHHTWTCTAAT (CS2_806R; reverse primer). An additional PCR reaction was performed for library preparation (Green et al. 2015).

16S rRNA gene sequencing analysis

Raw sequences reads were merged using the software package PEAR (Zhang et al. 2013) (v0.9.10). Low-quality sequences and chimeras were removed by the software package Mothur (Schloss et al. 2009) (v1.36.1).

The quality-controlled sequences were processed with the Quantitative Insights into Microbial Ecology (QIIME v1.9.1) package (Caporaso et al. 2010).

Briefly, sequence data were clustered into operational taxonomic units (OTU) at 97% similarity using UCLUST. Representative sequences from each OTU were extracted and aligned using PyNAST with a percent identity threshold of 60% to the Silva 16S rRNA bacterial database.

Statistical analysis

Alpha and beta diversity, including the observed species index, the community richness (Chao1) and diversity (Shanon and Simpson indices), principal coordinate analysis (PCoA), and ANOSIM statistical test were calculated for all samples using Quantitative Insights into Microbial Ecology (QIIME v1.9.1) package (Caporaso et al. 2010) (Tables S2 and S3, Fig. 4).

Climate data

Data regarding mean maximum and minimum air temperature, highest maximum air temperature, lowest minimum air temperature, monthly average precipitation (mm), and relative humidity (RH) was obtained from the local Sde-Boker Meteorology Station (30° 87' N, 34° 79' E) which is located about 10 km from the study site (source: Israel Meteorological Service) (Table S1).

Results

The results presented here provide initial insights into the structure and diversity of the microbial communities associated with the rock engravings panels during two seasons: winter 2017 and summer 2018.

Diversity analysis

From a total of 646,138 raw sequences reads that were obtained, an average number of 40,383 reads per sample passed the quality control set of sequence filters mentioned above, with mean lengths of about 450 base pairs.

The rarefaction analysis allowed comparison of species-richness (number of OTUs) between different rock samples. Rarefaction curves approached saturation after about 1500 sequences, suggesting that the majority of the operational taxonomic units (OTUs) were recovered in this study (Fig. 3).

The alpha diversity was estimated based on the observed species, Chao1, Shannon and Simpson indices and reflected the community diversity and species richness of each sample (Table S2). The observed species score of the bacterial communities ranged from 275 to 575 for winter samples and 527–741 for summer samples while the Chao1 index score ranged from 320 to 725 for winter samples and 590 to 886 for summer samples. According to these two indices, the samples obtained at summer reflected relatively higher bacterial richness. The Shannon scores ranged from 5.42 to 6.6 for the winter samples and from 5.93 to 6.6 for the summer samples. The Simpson scores ranged from 0.87 to 0.97 for the winter samples and 0.92 to 0.95 for the summer samples indicating that summer samples had relatively higher bacterial diversity. Nevertheless, these differences were not statistically significant.

Beta diversity analysis implementing principal coordinate analysis (PCoA) is presented in Fig. 4. The unweighted UniFrac distances show that compositions of these samples were clustered in two different groups, suggesting a difference between the two season samples. The weighted distances display a partial overlap between season samples. ANOSIM statistical test (Table S3) showed *P* value of 82% and *R*-value of –0.14 for the different season samples. Therefore, it was

demonstrated in this study that there was no significant difference in diversity between samples from winter 2017 and summer 2018. Thus, seasonal differences seem not to affect the bacterial communities' relative abundance but rather their composition (Table 1).

Taxonomy overview

DNA sequencing data revealed the presence of five main bacterial phyla, namely Cyanobacteria, Actinobacteria, Proteobacteria, Bacteroidetes, and Chloroflexi, across the analyzed sites. These main phyla were observed in samples from both winter and summer (Fig. 5, Table 1). All together, these phyla compromise about 85–95% of the rocks surface bacterial communities.

The predominant phylum was Cyanobacteria (10–60% relative abundance) followed by the Actinobacteria phylum (20–60% relative abundance), mainly represented by the orders Frankiales (0.02–38%) and Rubrobacterales (1–42%). These phyla were followed by the Proteobacteria phylum (5–35%) represented by the class Alphaproteobacteria (5–22%), the Bacteroidetes phylum (0.02–35%) represented by the order Cytophagales (0.4–2%) and then followed by the Chloroflexi phylum (1–12%). Within the Geodermatophilales order, members of the genus *Blastococcus*, *Geodermatophilus*, and *Modestobacter* were identified. The order Rubrobacterales was represented by the genus *Rubrobacter*. The phylum Cyanobacteria included members of the genus *Chroococcidiopsis* and of the genus *Microcoleus*. In addition, our study revealed approximately eight additional phyla, albeit at much lower percentages. These included Deinococcus-Thermus, Acidobacteria, Gemmatimonadetes, Planctomycetes, Firmicutes, Nitrospirae, and Verrucomicrobia. Five percent of the winter reads and 2.5% of the summer reads were unassigned (Fig. 5).

Fig. 3 Rarefaction curves of observed OTUs from rock samples: summer 2017 and winter 2018

Discussion

This study provides insights into the diversity and structure of the bacterial community associated with rock engraving sites at the Negev Desert (Israel) during two climatic different seasons. The winter is characterized by an average temperatures ranging from 14.8–19.1 °C during the day and –3.6–0 °C at night and by relative humidity of 68–77%. The summer is characterized by an average temperature ranging from 28.7–32.7 °C during the day and 5.5–12.5 °C during night and relative humidity of 49–65% (Table S1).

Culture-independent methods implementing Illumina-based deep sequencing were used in order to gain a broader understanding of these communities. Rarefaction curves of observed taxonomic units (OTUs) confirmed that implementing these methods revealed most of the taxonomic

Fig. 4 Unweighted (left) and weighted (right) principal coordinate analysis (PCoA) of the bacterial communities in winter (squares) and summer samples (dots)

data from all of the rock samples studied at the two seasons (Fig. 3). The analysis of the metagenome samples revealed five major phyla as the most common components of the microbiota at the sites studied during both seasons. These five main phyla were Actinobacteria, Cyanobacteria, Proteobacteria, Bacteroidetes, and Chloroflexi. Some phyla though present a marked variation in summer and in winter, as Actinobacteria, Proteobacteria, Bacteroidetes, and Chloroflexi, which are more abundant in winter (Table 1). Indeed, some phyla among them, Deinococcus-Thermus, Acidobacteria, Gemmatimonadetes, Planctomycetes, Firmicutes, Nitrospirae, and Verrucomicrobia, were found at much lower percentages in the samples (Fig. 5). Cyanobacteria were detected in all samples and are one of the major components of the bacterial communities, accounting for an average of 50% of all bacterial phyla obtained during the summer, and an average of 30% of all phyla reads obtained during winter. Cyanobacteria are already known as common photoautotroph bacteria inhabiting soil and rock surfaces in a wide variety of terrestrial habitats in hot and cold desert crusts (Friedmann 1980; Krumbein and Jens 1981; Wierchos et al. 2006; Dong et al. 2007). These organisms are considered as primary producers and generally adapted to resist adverse conditions due to their thick cell wall and the presence of protective pigments. Thus, the higher light levels

during summer may accelerate their colonization (Chrispim and Gaylarde 2005; Li et al. 2016; Chimienti et al. 2016). All this may explain their relatively higher abundance as was detected in our study in summer samples. For example, in our study, the Cyanobacteria was represented by the radiation-resistant *Chroococcidiopsis* sp., a genus that is frequently detected in stone monuments and rock sites in desert climatic zones as well as in Mediterranean climatic zones and beyond (Friedmann 1980; Büdel et al. 2004). The relative abundance of the Cyanobacteria in the summer samples may be either the result of more abundant Cyanobacteria or may be a result of the relatively lower abundance of other bacterial components as for example, Actinobacteria, in the rocks. It is likely that the presence and growth of many of the heterotrophic bacterial groups are dependent on their adaptation to high temperature and irradiance and low humidity. The heterotrophic Actinobacteria phylum detected in all the samples accounted for an average of 33% of the winter season bacterial communities and of 29% of the summer communities and are therefore considered in this study to be the second largest member of the bacterial communities. Members of this phylum were detected as a dominant bacterial taxon in subterranean environments in stone and rock cultural heritage sites in other geographic areas (Urzi et al. 2001). The Actinobacteria were also detected in several outdoor environments (Li et al. 2016). An explanation for the high fraction of Actinobacteria could be due to their strong cell walls and their capability for forming spores, characteristics that may allow their survival in harsh environments. In our study, the Actinobacteria were represented mainly by members of the orders Geodermatophilales (represented by the genera *Geodermatophilus*, *Blastococcus*, and *Modestobacter*) and by the order Rubrobacterales (represented by the genus *Rubrobacter*). These taxa were already known from previous studies regarding stone monuments and rock sites (Urzi et al. 2001; Li et al. 2016) indicating their ability to resist adverse environmental conditions such as ultraviolet light, ionizing

Table 1 Relative abundances of main bacterial phyla associated with the rock engraving panels

Phylum	Relative abundance (%) Winter sampling	Relative abundance (%) Summer sampling
Actinobacteria	17–58	23–37
Cyanobacteria	10–61	44–61
Proteobacteria	5–35	4–10
Bacteroidetes	1–35	0.02–2.5
Chloroflexi	2–12	0.07–5

Fig. 5 Bacterial phylum level taxonomic comparison between samples collected from the petroglyphs sites at two seasons: winter 2017 and summer 2018 (implementing 16S rRNA gene sequencing)

radiation, desiccation, and heavy metals. Similarly molecularly diverse group of the Actinobacteria genus *Rubrobacter* was reported in Australian dry soil (Chanal et al. 2006). Actinobacteria have also been found on the surface of the ancient sculptures in Yungang (Yan et al. 2012). Thus, the fact that Actinobacteria was found as one of the dominant phylum in the present study is not surprising especially when taking into account their competitive advantages such as their capacity for sporulation, their wide metabolic capacities, and their UV repair mechanisms (Thulani et al. 2015). A third major group, members of the phylum Proteobacteria, was detected in all samples and accounted for a mean of 13% of all winter season bacterial phyla and a mean of 8% of all summer phyla. This phylum was represented by the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria orders. Interestingly, these groups have been found in other rock sites from a wide geographic range, including the Atacama Desert and Black Canyon (Northup et al. 2010; Kuhlman et al. 2008). Other differences between summer and winter microbial diversity included differences in relative numbers of the members of the Chloroflexi phylum. They accounted for a mean of 6.6% of all winter season samples and a mean of 2.5% of all summer season samples. Members of this phylum were originally thought to live only in extreme environments such as hot springs but have been reported in temperate and even cold environments (Costello and Schmidt 2006). Indeed, they were also detected in rock varnish microbial community from the Yungay region of the Atacama Desert as well as rock varnish from the Black Canyon (Kuhlman et al. 2008 and Northup

et al. 2010). Some of the other taxa that were found in the present study were found in low abundance and present unique adaptation mechanisms to extreme environments. One example is given by the members of the order Deinococcales of the bacterial phylum *Deinococcus-Thermus*. These bacteria have thick cell walls and unique resistance to exposure to high doses of gamma and UV radiation. This resistance to environmental hazards represents a trait of Terrabacteria, a well-supported phylogenetic group composed of Actinobacteria and four other major lineages of eubacteria (Firmicutes, Cyanobacteria, Chloroflexi, and *Deinococcus-Thermus*) that are assumed to colonize land and terrestrial environment billions of years ago. A study of an arid soil collected from the Sonoran Desert yielded 60 strains of the genus *Deinococcus* that were subsequently assigned to nine novel species (Rainey et al. 2005). This strengthens the premise that the rock surfaces may be considered an oligotrophic substrate. The arid climate appears to put some selective environmental pressure limiting the diversity of the microbial communities and enabling the survival of specific organisms adapted to these arid zone conditions. When examining the abundance of the bacterial communities during the different seasons at all taxonomy levels, a great deal of similarity between the two season samples was observed. Principal coordinate (PCoA) indices and ANOSIM test results pointed out that there were no significant differences in bacterial diversity between the winter and summer samples (Fig. 4, Table S3). This may be explained by the fact that rock varnish and the surface layers play a role as shelter from the

extreme environment conditions, enabling stable living conditions (Golubic et al. 1981; Wierzbos et al. 2012). Thus, our findings present initial insights into specific bacterial communities adapted to the climatic conditions and the specific petrographic characteristics.

Conclusion

Our results demonstrated that implementing next-generation sequencing (NGS) methods provides access to a larger group of bacterial taxa and therefore is an important first step in the study of the microorganisms inhabiting the rock art sites. In the present study, we showed that although differences were found between seasons, they were not significant. Furthermore, there was not a significant difference in the diversity of the taxa across samples from both seasons though there was a difference in richness and abundance. Future investigation of the presence and distribution of microbiota at higher taxonomic levels (e.g., genus or species level) will likely provide a deeper understanding of possible differences in diversity during summer and winter. We therefore suggest that further study implementing microclimatic measurements of the rock habitat will expand our knowledge regarding the influence of environmental factors on the diversity and functionality of the bacterial communities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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