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Analysis of bacterial communities and characterization of antimicrobial strains from cave microbiota

Muhammad Yasir

King Abdulaziz University, King Fahd Medical Research Center, Special Infectious Agents Unit, Jeddah, Saudi Arabia

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ABSTRACT

In this study for the first-time microbial communities in the caves located in the mountain range of Hindu Kush were evaluated. The samples were analyzed using culture-independent (16S rRNA gene amplicon sequencing) and culture-dependent methods. The amplicon sequencing results revealed a broad taxonomic diversity, including 21 phyla and 20 candidate phyla. *Proteobacteria* were dominant in both caves, followed by *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, and the archaeal phylum *Euryarchaeota*. Representative operational taxonomic units from Koat Maqbari Ghaar and Smasse-Rawo Ghaar were grouped into 235 and 445 different genera, respectively. Comparative analysis of the cultured bacterial isolates revealed distinct bacterial taxonomic profiles in the studied caves dominated by *Proteobacteria* in Koat Maqbari Ghaar and *Firmicutes* in Smasse-Rawo Ghaar. Majority of those isolates were associated with the genera *Pseudomonas* and *Bacillus*. Thirty strains among the identified isolates from both caves showed antimicrobial activity. Overall, the present study gave insight into the great bacterial taxonomic diversity and antimicrobial potential of the isolates from the previously uncharacterized caves located in the world's highest mountains range in the Indian sub-continent.

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Introduction

Caves with surface entrances represent one of the unique and poorly studied ecosystems on Earth. They include hydrological systems that are relatively isolated from the surface, and share basic physicochemical conditions, including complete darkness, constant humidity, and thermal stability.[1,2] Caves comprised of unique underground communities of organisms, and cave microclimates often support dense populations of extremophiles.[3] Multidisciplinary studies on cave microbiology have implicated microorganisms in the geological processes of caves, and have opened several avenues of research, including cave geochemistry, cave environmental microbiology and identification of new microbial species and exploration of novel biotechnological molecules from cave sources.[4,5] Recently, several new members of the genera *Catellatospora* and *Nonomuraea* were discovered in caves of Mexico

E-mail: yasirkhattak.mrl@gmail.com
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and Northern Thailand.[6,7] In 2005, *Agromyces subbeticus* sp. nov., was isolated from a cave in the Cordoba area of southern Spain.[8] Jurado et al. reported *Aurantimonas altamirensis* sp. nov., a Gram-negative member of the order *Rhizobiales* from Altamira Cave, located in the Cantabria, Spain.[9] These microorganisms newly discovered in caves demonstrate the potential for identifying secondary metabolites that have yet to be fully evaluated and exploited.[4,7]

Caves are found worldwide, and biospeleological research has been much increased in the last twenty years.[2,4] Available literature indicates the existence of short caves in the world's highest mountains range called Karakoram, Hindu Kush, and Pamir, which incorporate some of the world's highest peaks, including the K2 (8610 m) and Nanga Parbat (8125 m) located in the sub-continent.[10] In the Chitral area of Pakistan, the Hindu Kush mountain is surrounded by a high limestone plateau, and there are various unverified reports of caves in this region.[11] The Nanga Parbat has Rakhiot Cave, which is 73.2 m long and the highest known cave at an altitude of 6644 m.[11] So far, no systematic studies have been conducted to explore the caves system in those mountains. The caves identified by local people have never been surveyed for microorganisms. In this study for the first time, microbial communities were investigated in the two caves, Koat Maqbari Ghaar (KMG) and Smasse-Rawo Ghaar (SRG), located in the Hindu Kush Mountain and situated in the northern Khyber Pakhtunkhwa province of Pakistan. Advances in sequence-based metagenomic approaches have made studies on microbial diversity and community composition in diverse environments more possible and informative. However, using high-throughput sequencing techniques alone to study the microbial community in an environment has the possibilities to miss the low abundant taxa as previously observed.[12] In this study, an integrated approach of culture-based and culture-independent pyrosequencing methods were used to gain a more accurate representation of the microbial communities in the study sites. Bacterial strains isolated in this study were screened for antibacterial and antifungal activities.

Materials and methods

Samples collection and chemical analysis

This study represents a polyphasic analysis of microbial communities from two caves in the Hindu Kush mountain range situated in the North-West region of Pakistan. The local names of those caves are Koat Maqbari Ghaar (KMG, 34°49′13.06″ N, 72°30′41.81″ E) and Smasse-Rawo Ghaar (SG, 34°33′38.1″ N, 71°51′03.4″ E), and they are located in the Swat and Malakand division, respectively. The KMG cave is about 2.5 m wide and 15 m deep, and is located at about 1260 m above sea level. The SRG cave has an almost horizontal orientation and around 2 m wide, 10 m long, and is located at 1062 m above sea level. Both caves are of dried nature and very limitedly influenced by anthropogenic activities. Sediment samples in three replicates were collected from each cave using autoclaved and sterilized bottles. The samples were collected in February 2013. All of the samples were stored at 4 °C for around 20 h during transportation, and were immediately processed for culturing after arriving in the laboratory. A part of each sample was stored at −80 °C for metagenomic DNA extraction. No specific permission was required for sampling the studied caves. These lands were not privately owned or protected in any way and the caves are not part of a national park or reserve. Our sampling did not involve endangered or protected species.

The pH of each sample was measured using Sartorius pH meters (Denver, Germany) in a 1/10 (w/v) saturated colloid solution of sediment in deionized water. Temperature was measured using ASTM thermometers (Gilson, USA) on each site. Total soil organic matter and total nitrogen were determined using the partial oxidation method and micro Kjeldahl method.[13] Total phosphorus was measured colorimetrically.[13] Physicochemical analysis were performed in triplicate for each sample.

DNA extraction and pyrosequencing

Total DNA was extracted from each homogenized cave sediment replicates using the protocol for the PowerSoil® DNA extraction kit (Mo Bio Laboratories, Carlsbad). Amplification of the 16S rRNA gene hypervariable region V4 was performed using bar-coded 515F and 806R universal primers containing A and B sequencing adaptors, following the procedure previously described.[14] PCR products were quantified using high-sensitivity Qubit technology (Invitrogen, USA), and were purified using Agencourt Ampure beads (Agencourt, USA). The 454 FLX–titanium pyrosequencing platform (Roche, Basel Switzerland) was used to perform high-throughput sequencing following the manufacturer's protocol. Raw pyrosequencing data was processed using the analysis pipeline of MR DNA (Texas, USA).[14] Sequence reads <150 bp were removed, and the remaining reads were screened for homopolymer runs exceeding 6 bp, chimeric sequences, and sequences containing Ns; all of these were also excluded. Barcodes and primers were depleted from sequences. High quality sequence reads were clustered into OTUs using a threshold of 97% sequence similarity. For singleton reads the default value of 2 reads in QIIME v1.9 software was used to exclude from further analysis.[15] OTUs were taxonomically classified using BLASTn against the curated databases GreenGenes, RDP (http://rdp.cme.msu.edu), and NCBI (www.ncbi.nlm.nih.gov).[16] The alpha diversity analysis was performed with Chao1 and the non-parametric Shannon formula using QIIME v1.9 software.[15]

Culture-dependent samples processing

Sediment samples were serially diluted for the isolation of bacterial colonies with improved culture methods, based on an increased number of inoculation plates for each dilution, longer incubation times, selection of micro-colonies, and use of modified culture media as previously described.[17] Briefly, two different concentrations of R2A medium (full strength 18 g/L and half strength 9 g/L) and diluted nutrient broth (1/5 strength 3 g/L and 1/10 strength 1.3 g/L) supplemented with 1.5% agar, 20% aqueous extract of the collected cave sediment, and two incubation conditions, 17 °C and 37 °C, were used to culture bacteria from the caves samples. The plates were incubated in aerobic condition for one week in seal

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