Europlanet TA Scientific Report

PROJECT LEADER

Project number: 20-EPN2-078
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TA Facility visited: Center for Microbial Life Detection (AT)

Project Title: Metagenomic analysis of the outstanding moonmilk speleothems from Grotta Nera, Majella National Park

Scientific Report Summary.
(plain text, no figures, maximum 250 words, to be included in database and published)

This project focused on the analysis of three samples from the Black cave (Grotta Nera) located in Majella Park (Abruzzi region, Italy). This cave presents outstanding calcitic moonmilk structures that are unique in the World in terms of both abundance and dimension. Metagenomic and metabolomic analyses of three samples (A1, apical; A2, lateral; A3, core) collected from one of the moonmilk speleothem from Grotta Nera, were performed. The DNA was extracted using the DNA powersoil kit (Qiagen) modified to include a bead-beating step with MagNA lyser (Roche) for the initial sample treatment. MG-RAST was used to analyse the metagenomic data considering both the taxonomy composition and the functional categories (KO categories). The taxonomy composition of the metagenomic sequences indicated that the dominant phyla were *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Acidobacteria*, and *Verrucomicrobia*. *Actinobacteria* were more abundant in the A1 and A2 as compared to the A3 sample, while in A3 *Proteobacteria* (in particular, *Betaproteobacteria*) was enriched as compared to other two samples. The metabolomic analysis was carried out using NMR, extracting the metabolites from 100 mg of each sample (in triplicate). The results indicated that in A2 and A3 samples were enriched by specific metabolites (glycerol in A3 and alanine, acetate, ethanolamine and 3-hydroxybutirate are enriched in A2) suggesting distinct metabolic activities in the microbial communities of these two samples.

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This project focused on the analysis of three samples from the Black cave (Grotta Nera) located in Majella Park (Abruzzi region, Italy). This cave presents outstanding calcitic moonmilk structures that are unique in the World in terms of both abundance and dimension (Cacchio et al. 2016). Moonmilk is a secondary speleothem usually occurring on cave walls and ceiling and typically consisting of assemblages of microcrystalline carbonate minerals with spongy to powdery appearance. Some investigations suggest that microbial activities contribute to moonmilk development and maintenance (Barton and Northup, 2007). The aim of this Europlanet project was to perform metagenomic analyses of three samples collected from one of the moonmilk speleothem from Grotta Nera. These three samples corresponded to the apical bottom part of the speleothem (samples "A1"), the external lateral part (samples "A2"), and the inner part or moonmilk core (samples "A3"). DNA was extracted using the DNA powersoil kit (Qiagen) modified in order to include a bead-beating step with MagNA lyser (Roche) for the initial sample treatment.
The metagenomic sequencing was performed by Macrogen using Illumina NovaSeq 6000 with a configuration of 2 x 150 bp with an output of 50 Mio (7.5 Gb) per sample. The number of reads that were obtained from each sample are reported in Table 1 along with the total amount of DNA that was extracted from each sample under analysis.

Table 1 Description of the samples and the sequencing data

Sample name	Moonmilk zone	Total amount of DNA (quantified by Macrogen)	Number of reads obtained
A1	Bottom	500 ng	4,371,100
A2	External	5226 ng	42,056,690
A3	Inner	66 ng	3,754,554

MG-RAST was used to analyse the metagenomic data considering both the taxonomy composition and the functional categories (KO categories). The taxonomy composition of the metagenomic sequences is shown in Figure 1. The most dominant phyla were Proteobacteria, Actinobacteria, Firmicutes, Planctomycetes, Acidobacteria, and Verrucomicrobia. By comparing the relative abundance of these dominant phyla among the three samples we could notice that Actinobacteria were more abundant in the A1 and A2 as compared to the A3 sample. In A3, Proteobacteria was enriched as compared to other two samples. By considering the Proteobacteria classes, A3 was enriched of Betaproteobacteria, while A2 was enriched of Gammaproteobacteria.

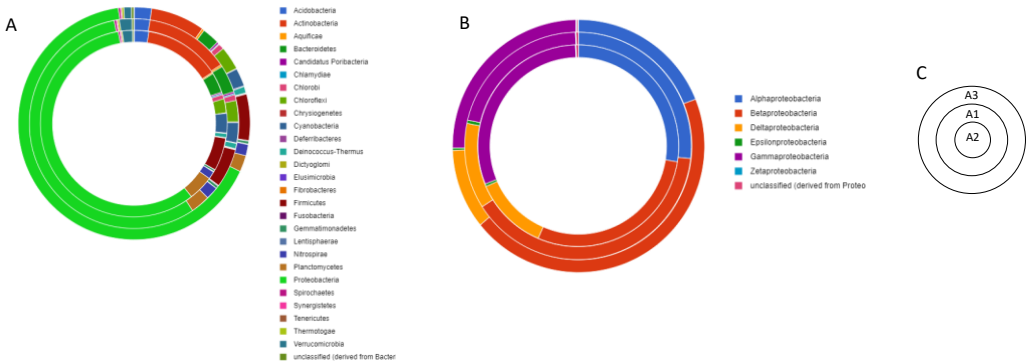

Figure 1 Taxonomy composition of the reads obtained from the metagenomic sequencing of the samples A1, A2, A3. A) Relative abundance of the phyla; B) Relative abudance of the Proteobacteria classes; C) Indication of the samples order in panels A and B

In regard with the functional categories, a first analysis regarded the carbohydrate, amino acids, and energy metabolism (Fig. 2). The three samples showed similar abundance profile for each category with glycolysis, glyoxylate and dicarboxylate metabolism, pentose

phosphate and pyruvate metabolism being the dominant categories in carbohydrate metabolism. Alanine, aspartate and glutamate metabolism was the most abundant in the amino acid metabolism category together with the glycine, serine and threonine metabolism. Oxidative phosphorylation was the dominant energy metabolism followed by methane metabolism.

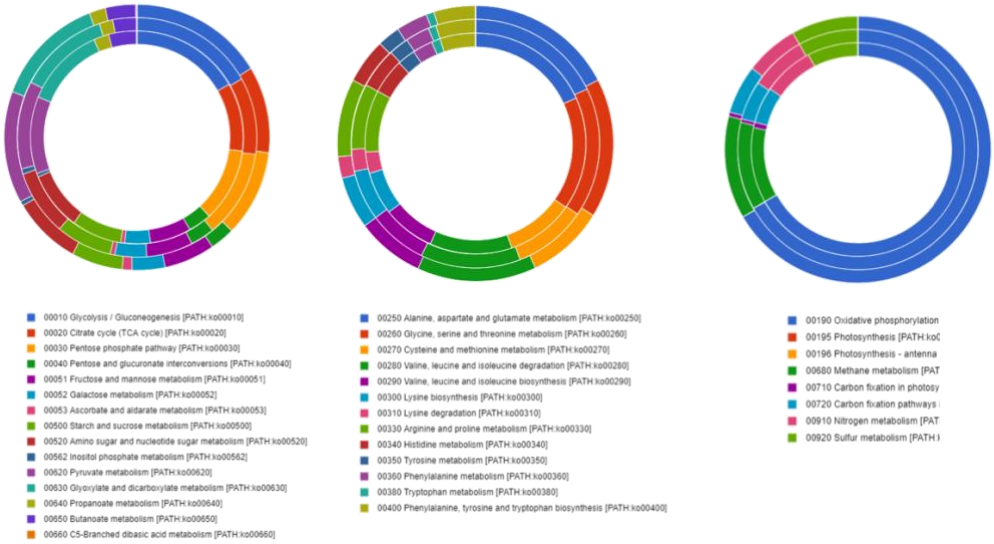

Figure 2 Functional categories of the reads obtained from the metagenomic sequencing of the samples A1, A2, A3. Left panel: Carbohydrate metabolism, middle panel: Amino acid metabolism, Right panel: Energy metabolism.

The similarity among the three samples by comparing the main metabolic categories (level 2 of KO categories in MG-RAST) suggest that the main metabolic processes are conserved among the microbial communities enriched in each moonmilk niche (external, apical and care). However, further analyses will be carried out to get insights into possible differences in specific metabolic/enzymatic functions.

NMR-based metabolomic analysis was also performed on 100 mg of each sample (analysed in triplicate) that was extracted with Precellys beads in H_2O/MetOH (Zhou et al. 2021). The surnatant was liophylized and dissolved in NMR buffer for analysis (Zhang et al. 2021). The NMR analyses were carried out by Dr Tobias Madl and Dr Hansjörg Habisch (Medical University of Graz) and the metabolite detection singals are shown in Figure 3.

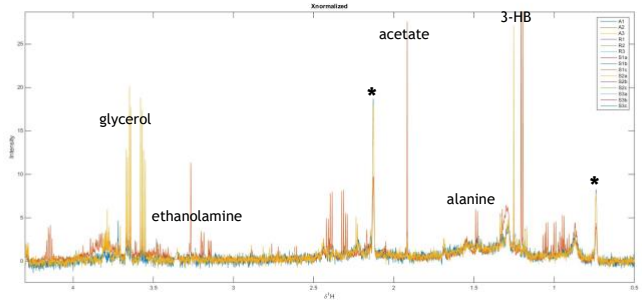

Figure 3 Overlay of ^{1}H NMR spectra of A1, A2 and A3 samples. Other samples are also indicated (R and S code) however the only metabolites that are significantly enriched in each of the three A sample are indicated.

The result of the metabolomic analysis indicated that the only A2 and A3 samples were enriched by specific metabolites. The metabolites alanine, acetate, ethanolamine and 3-hydroxybutirate are enriched in A2, while glycerol was enriched in A3. These indications suggest the presence of metabolic activities that are distinctive for the samples A2 and A3.

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Zhou Q, Kerbl-Knapp J, Zhang F, Korbelius M, Kuentzel KB, Vujić N, Akhmetshina A, Hörl G, Paar M, Steyrer E, Kratky D, Madl T. Metabolomic Profiles of Mouse Tissues Reveal an Interplay between Aging and Energy Metabolism. Metabolites. 2021, 12(1):17.

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- Give details of any publications arising/planned (include conference abstracts etc)

- Host confirmation

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Dates for travel to accommodation for TA visit (if physical visit by applicant)	Start Date of TA project at facility	Number of lab/field days spent on TA Visit pre-analytical preparation	Number of days in lab/field site for TA Visit	Number of days spent in lab for TA Visit data analysis	End Date of TA project at facility	Dates for travel home (if physical visit by applicant)
Departed: 04-10-2021 Arrived: 04-10-2021	04-10-2021	1	4	2 (virtual)	26-04-2022	Departed: 07-10-2021 Arrived: 07-10-2021

The host is required to approve the report agreeing it is an accurate account of the research performed.

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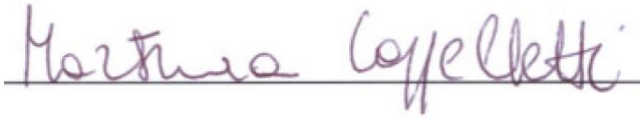
Project Leader Signature	
Date	**05.06.2022**