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Short Communication

Preliminary assessment of methanogenic microbial communities in marine caves of Zakynthos Island (Ionian Sea, Greece)

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Abstract

Mediterranean marine caves remain largely unexplored, while particularly limited information is available about microbial life in these unique environments. This study is a preliminary assessment of the composition of the active anaerobic microbial community colonizing the walls of newly explored systems of underwater caves and small cavities in Zakynthos Island. The interior of these caves is densely coated with egg-shaped, foam-shaped and filamentous biological structures that are characterised by a strong odour of hydrogen sulphide gas. A total of twelve structures scrapped from cave rocks were subjected to anaerobic cultivation for up to 208 days. Strong to moderate methanogenesis was observed in two different types of egg-shaped structures and one foam-like structure. Interestingly, this was observed in experiments that were performed at room temperature (i.e. 25°C), which is substantially lower than those typically considered optimum for methane production (e.g. 35°C). An analysis of the 16S rRNA genes revealed a clear dominance of archaea and bacteria closely related to known methane producers and sulphate reducers, including members of the families *Methanomicrobiaceae, Desulfobulbaceae*, *Desulfobacteraceae*, *Desulfuromonaceae*, *Campylobacteraceae*, *Marinifilaceae*, *Clostridiaceae*, Incertae Sedis – Family I & II. These results show that Mediterranean marine caves can host members of archaea and bacteria with potential biotechnological interest that deserves further investigation.

Keywords: 16S rRNA gene analysis; Anaerobic cultures; Mediterranean marine caves; Methanogens; Sulfate reducers.

Introduction

Around the world, marine caves constitute biodiversity hotspots supporting unique diversity elements and species-rich communities of great ecological, scientific and conservation value (Gerovasileiou *et al.*, 2016). Recent biodiversity assessments of this habitat type revealed that Mediterranean marine caves harbour exceptionally high biodiversity (>2000 taxa belonging to 58 major taxonomic groups), including several rare and protected species (Gerovasileiou & Voultsiadou, 2012, 2014; Gerovasileiou *et al.*, 2015). However, insights into their microbiology are limited. Specifically, only 6 marine caves have been investigated regarding their microbial communities, namely, Grotta Azzurra and Grotta Sulfurea on the Tyrrhenian coasts of Italy (Mattison *et al*., 1998; Canganella *et al*., 2002; 2007; Maugeri *et al*., 2010); Gymnasium, Granchi and Mazzere caves on the Ionian coast of Sicily (Guido *et al*., 2012, 2013, 2014, 2016; Sanfilippo *et al*., 2015); and Kakoskali cave in Cyprus (Guido *et al*., 2017). However, only the former two have been investigated for their microbiota, using molecular techniques, while studies on the other three caves involved the morphological identification of microbial structures.

Methane is a key component of the carbon cycle in many aquatic environments and it is an important end product in the anaerobic degradation of organic matter in marine and freshwater sediments, particularly in zones where more energy-yielding oxidants, such as sulphate, nitrate, or ferric iron, are depleted (Heyer, 1990). Cave ecosystems belong to this type of environments that can host methanogens, i.e. microorganisms that can gain en-

ergy from the conversion of a limited number of simple substrates into methane (Engel, 2010). During a recent investigation concerning marine cave biodiversity in the National Marine Park of Zakynthos – NMPZ (eastern Ionian Sea, Greece), cave and cavity systems densely colonized by egg-shaped, foam-shaped and filamentous biological structures were discovered. These systems were also characterized by a strong odour of hydrogen sulphide, which pointed to the presence of anaerobic activity. The latter is a fundamental prerequisite for the growth of methanogenic microorganisms, as they are all known to be obligate anaerobes. A number of biological samples, representing different morphological types of colonies, were collected from the caves and used for the preparation of anaerobic enrichment cultures. The latter were subsequently subjected to 16S ribosomal RNA gene analysis in order to obtain a preliminary assessment of the active sulphate reducers and methane producers, and evaluate their potential in the fields of bioenergy and bioremediation.

Materials and Methods

Sampling site

During a sampling campaign that took place in June 2015, microbial colonies were collected from marine cave, cavity and fissure systems (semi- and entirely submerged) located at a depth range of 0-25 m, on the south-western coasts of Zakynthos Island (37.661° N, 20.860° E), within the National Marine Park of Zakynthos (Fig. 1a). Representative samples of six different types of biological colonies were collected from cave walls (two replicates) by SCUBA diving, placed in sterile caps at room temperature (total of 12 samples; Supplementary Fig. S1), transferred to the laboratory and immediately subjected to a series of cultivation experiments to enable enrichment of the anaerobic bacteria capable of producing methane (Supplementary Fig. S2). No particular pattern was identified with regard to the distribution of the different types of colonies in the studied caves.

Enrichment and isolation of methanogenic microbes

Wolfe's anaerobic medium was selected for the enrichment and isolation of methanogenic microbial communities. Technical details about the preparation of this complex anaerobic medium, the transfer of medium aliquots into serum bottles under anaerobic conditions and the cultivation of methanogenic microbes are thoroughly described in Wolfe (2011) and are presented in Supplementary Materials & Methods. For the enrichment of methane-producing microorganisms, a total of 12 cultures were prepared in 100-mL serum bottles that

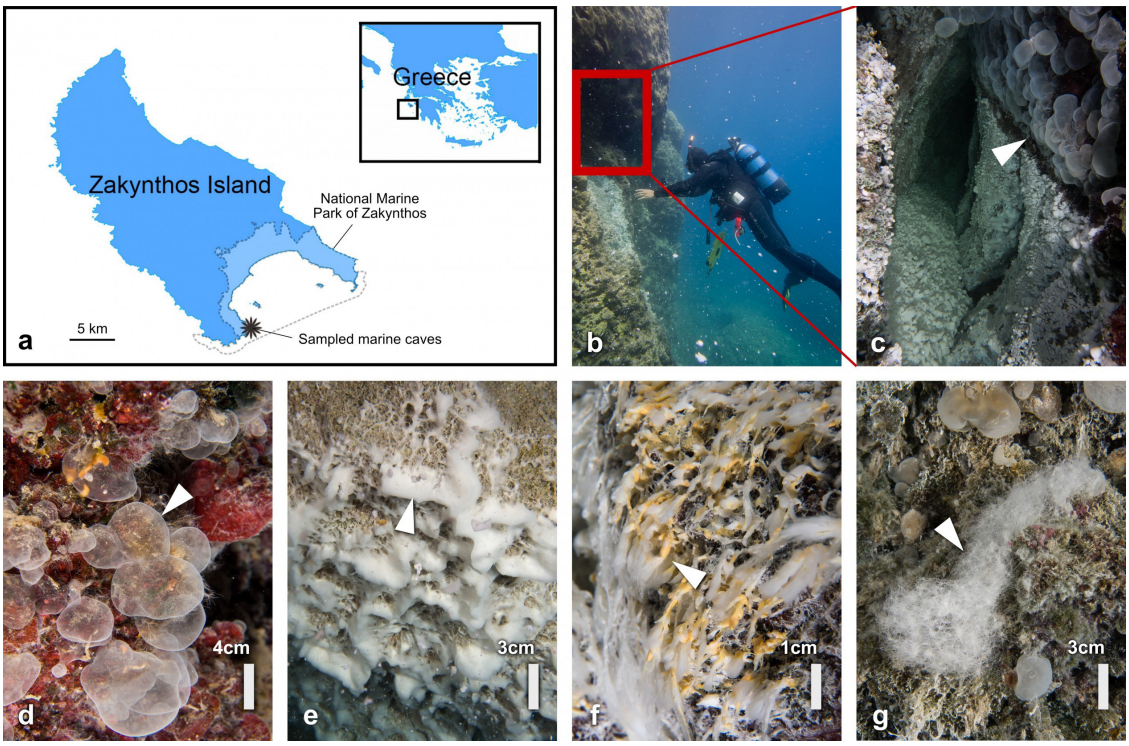

Fig. 1: a) Map showing the geographic location of the studied marine caves in the National Marine Park of Zakynthos, Greece; b-c) crevice colonized by methane producing egg-shaped and foam-like microbial structures; d) egg-shaped, e) foam-like and f-g) filamentous microbial structures found in the study area. Microbial structures are indicated by white arrows. (Photographs by T. Dailianis).

were inoculated with pieces of each biological structure (two replicates). The bottles were tightly sealed with Teflon-lined crimp caps to maintain anaerobic conditions and incubated inverted at room temperature (i.e. 25°C) in the dark (Supplementary Table S1). Production of methane was regularly assessed in all cultures by withdrawing headspace samples (100 μL) from the serum bottles using a gas-tight syringe and analyzing them on a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (GC-FID) and a Carboxen 1006 PLOT capillary column (30 m × 0.53 mm; Supelco, Inc.).

16S rRNA gene and phylogenetic analysis

The microbial methanogens enriched from the collected samples were characterized by cloning and sequencing of PCR-amplified 16S rRNA genes following the experimental procedure described by Polymenakou *et al*. (2005). Details about this procedure are provided in Supplementary Materials & Methods. The enrichment culture showing the highest methanogenic activity was selected for the extraction/isolation of genetic material and the amplification of the highly conserved ribosomal RNA gene (16S rDNA), which is typically used as a marker for the identification of microbial species. The sequencing reactions were undertaken using the 20-bp forward primer for bacterial clones Bac-27f 5'-AGAGTTTGATCCTGGCTCAG-3' (Lane, 1991) and the 16-bp forward primer for archaeal clones Arch-8f 5'-TCCGGTTGATCCTGCC-3' (Teske *et al.,* 2002). A total of 83 bacterial and 10 archaeal clones were successfully characterized during this investigation and were used for phylogenetic analysis. Approximately 600 bp long parts of 16S rDNA sequences were aligned by ClustalW (Larkin *et al.*, 2007). At this stage, identical sequences (i.e. sequences showing 100% similarity) were grouped into unique operational taxonomic units (OTUs) and were indicated as Bacterial OTU A-K and Archaeal OTU A-C (Supplementary Table S2). Phylogenetic analysis was performed by Bayesian statistics via MrBayes (Ronquist & Huelsenbeck, 2003) and tree topology was visualized via iTOL (Letunic & Bork, 2016). The 93 partial 16S rDNA sequences generated within the framework of this study were deposited in GenBank under accession numbers MF627323-MF627415.

Results

The investigated marine cave systems of Zakynthos Island were characterized by the presence of different morphological biological structures forming small egg-shaped (2 types of colonies), foam-like (2 types of colonies) and filamentous mats (2 types of colonies), and pervaded by a strong odour of hydrogen sulphide gas, which is typically indicative of prevailing anaerobic conditions (Fig. 1; Supplementary Fig. S1). Out of 12 samples investigated over the course of a 208-days cultivation period, the two different types of egg-shaped structures (coded as 1A/1B and 4A; Fig. 1; Supplementary Figs. S1, S2 and Table S1) and one type of foam-like structure (coded as 3A) (Figs. S1) indicated strong or moderate methanogenesis under an atmosphere of H_2:CO_2 (up to 30% v:v methane in the headspace was measured).

Of the 93 bacterial and archaeal clones that were sequenced during this investigation, a total of 51 bacterial and 3 archaeal OTUs were identified and assigned to nine families (Fig. 2; Supplementary Table S2). More specifically, the microorganisms that dominated the enrichment anaerobic culture showing high methanogenesis were closely related to archaeobacterial strains of family *Methanomicrobiaceae*, and to bacterial strains of families *Desulfobulbaceae*, *Desulfobacteraceae*, *Desulfuromonaceae*, *Campylobacteraceae*, *Marinifilaceae*, *Clostridiaceae*, Incertae Sedis – Family I & II. Interestingly, two bacterial clones did not have any cultured representatives and formed a new cluster named "*ZakynCa"*.

Discussion

Mediterranean marine caves are largely unexplored and only recently have been recognized as important biodiversity reservoirs that deserve further investigation (e.g. Gerovasileiou & Voultsiadou, 2012; Guido *et al.,* 2016). Regarding their microbiology, only few submarine caves on the Ionian coast of Sicily and Cyprus have been morphologically investigated. The submerged marine caves of Sicily are dominated by unique bioconstructions, named biostalactites, which are constituted of small serpulids and other metazoans associated with carbonate micrites (Guido *et al.,* 2012, 2013, 2014, 2016; Sanfilippo *et al.,* 2015). In these serpulid bioconstructions, micrite precipitates are probably induced by the presence of sulphate reducing bacteria (Guido *et al.,* 2012). Similar serpulid bioconstructions have been observed recently in submarine caves of Lesvos Island, North Aegean Sea (Sanfilippo *et al.,* 2017) and the Kakoskali submarine cave of Cyprus (Guido *et al.,* 2017). However, such bioconstructions were not found in the surveyed caves of Zakynthos. Extensive microbial biofilms/mats have also been found on the rocky surfaces and even on the bottom sediment of two submarine caves with internal sulphur springs, namely, Grotta Azzurra and Grotta Suflurea in the Tyrrhenian Sea, Italy (Canganella *et al.,* 2002, 2007), which support a unique chemosynthetic ecosystem (Southward *et al.,* 1996).

The current study presents a preliminary assessment of the active microbial anaerobes residing in the biological structures covering the walls of newly discovered systems of submarine caves and cave-like formations in Zakynthos. Anaerobic enrichment cultures performed during this study indicated strong methanogenesis only in egg-shaped and one foam-like microbial structure.

Sequencing analysis of the 16S rRNA genes of collected microorganisms revealed the dominance of archaea and bacteria closely related to known methane producers of the genus *Methanoculleus* and sulphate reducers

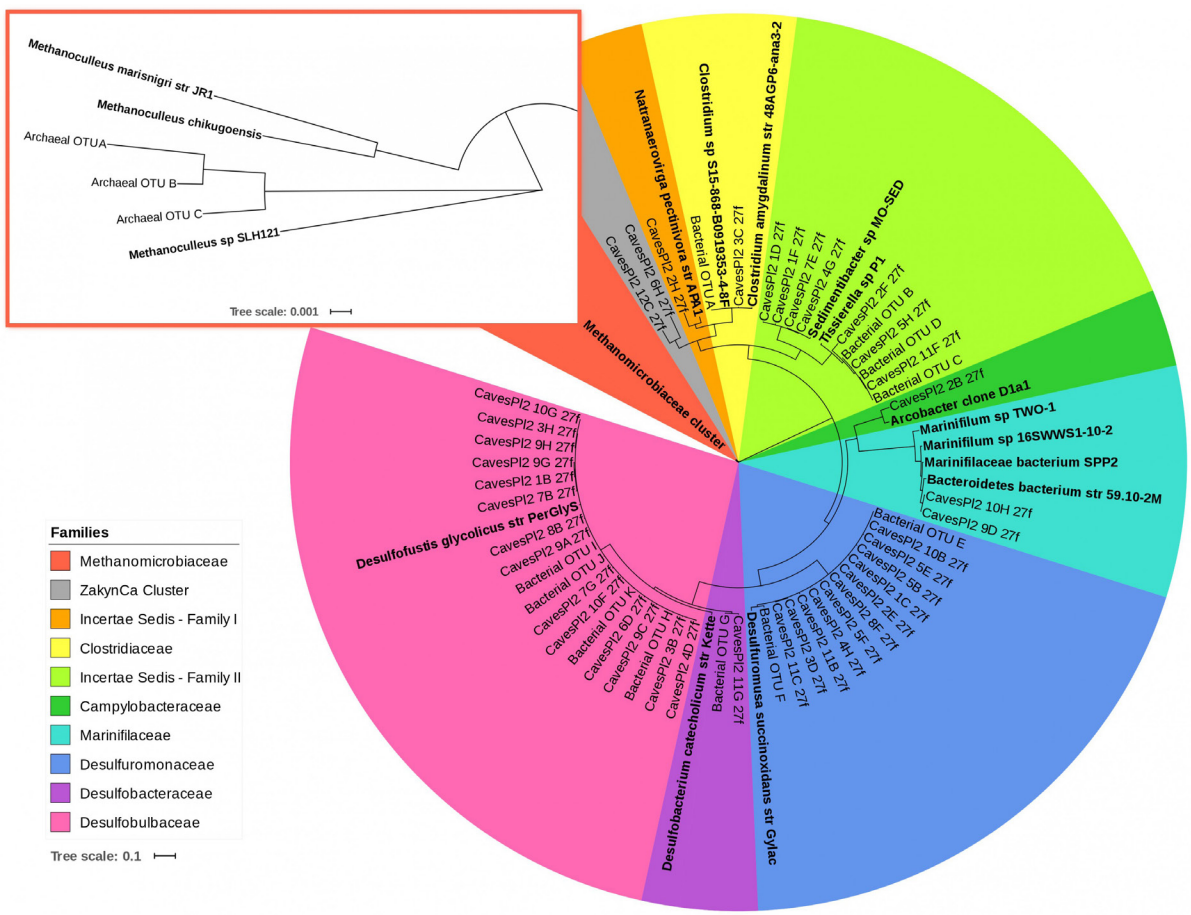

Fig. 2: Phylogenetic tree of microbial communities in methane enrichment culture of all sequenced clones. Insert: Details of *Methanomicrobiaceae* cluster at 0.001 scale. Cultured close relatives are presented in bold. Branch lengths represent number of substitutions per site.

of the genera *Desulfuromusa* and *Desulfofustis*. All of the archaeal clones were closely related to the following three strains of *Methanoculleus* genus: i) *Methanoculleus sp. SLH121* isolated from the extreme environment of Haakon Mosby mud volcano (GenBank accession number: KC893306), ii) *Methanoculleus chikugoensis* isolated from paddy field soil in Japan (Dianou *et al.,* 2001; Oren, 2014) and iii) *Methanoculleus marisnigri* isolated from Black Sea sediment (Romesser *et al.,* 1979; Maestrojuan *et al.,* 1990; Oren, 2014). Members of the genus *Methanoculleus* are among the dominant methanogenic archaea found in biogas-producing reactor systems fed with renewable primary products (e.g. Maus *et al.,* 2012). Therefore, the discovery and isolation of new methanogens during the course of this study could be used in such reactors. Thus, our findings are of great commercial importance due to the possibility of producing artificial biogas, a renewable and environmentally compatible energy source (e.g. Jaenicke *et al.,* 2011; Maus *et al.,* 2012). Indeed, this is why most Mediterranean marine caves, characterized by anaerobic activity are deemed to be of potential biotechnological interest.

Together with the archaeal sequences most closely related to genus *Methanoculleus*, many different anaerobic bacterial communities were also identified. The vast majority of the bacterial sequences were closely related to the sulphur-reducing bacterium *Desulfuromusa succinoxidans* (Liesack & Finster, 1994) and to the sulphate-reducing bacterium *Desulfofustis glycolicus* (Friedrich *et al.*, 1996). Sulfate-reducing bacteria (SRB) are important in bioremediation procedures (Barton & Fauque, 2009). Recent studies have demonstrated the potential of SRB to be used in the bioremediation of BTEX-contaminated soils (i.e. benzene, toluene, ethylbenzene and xylene). In addition, SRBs have been shown to reduce 3-chlorobenzoate, chloroethenes or nitroaromatic compounds efficiently, and the usage of such strains has been proposed for the bioremediation of environments contaminated by trinitrotoluene and polychloroethenes. Bioremediation of toxic metals, such as U(VI) and Cr(VI), and of various sulphate-containing compounds, as well as the recovery of precious metals from waste streams (e.g. platinum,

palladium and gold) are some other important environmental remediation applications of SRBs (Barton & Fauque, 2009; Hussain *et al.,* 2016).

Overall, the newly discovered marine caves of Zakynthos host members of archaea and bacteria with potential biotechnological interest that deserve further investigation. In this preliminary study, we used enrichment cultures to characterize methanogenic microbial communities. Interestingly, strong to moderate methanogenesis was observed in experiments that were performed at room temperature (i.e. 25°C), which is substantially lower than those typically considered optimum for methane production (e.g. 35°C; Sánchez *et al.,* 2000). The ability of the isolated microbial communities to produce methane at the energetically less expensive ambient temperature implies potential large-scale exploitation. Finally, we would like to emphasize the necessity of an extensive survey on the chemistry/microbiology of Mediterranean marine caves and the application of modern molecular tools to unravel the metabolic capabilities of cave microbiomes and evaluate their potential applications in biotechnology.

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