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Supplementary Data

Methanogenic microbial communities in marine caves of Zakynthos Island (Ionian Sea, Greece)

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Materials and Methods

Enrichment and isolation of methanogenic microbes

This Wolfe's anaerobic medium that was used for the enrichment of methanogenic microbes contained the following ingredients (g/L): NH_4Cl , 1; NaCl , 0.6; NaHCO_3 , 5; KH_2PO_4 , 0.3; K_2HPO_4 , 0.3; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.16; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.009; resazurin 0.1% solution, 1 ml; cysteine \cdot HCl 200mM, 15ml; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ 200mM, 4ml; 1 ml of vitamin-mix solution containing 10 mg/L of nicotinic acid, p-aminobenzoic acid, calcium pantothenate, pyridoxine, riboflavin, thiamine and 5 mg/L of biotin, folic acid, α -lipoic acid, B12. Moreover, 1 ml was added from a trace element solution consisting of the following ingredients (g/L): trisodium nitrilotriacetic acid, 1.5; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 0.8; NaSeO_3 , 0.2; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; H_3BO_3 , 0.01; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01. For the isolation of methane-producing microorganisms, a total of 12 cultures were prepared using specialized glass bottles to maintain anaerobic conditions (120 ml glass serum bottles capped with viton stoppers and aluminum crimp seals). The preparation of the medium was held under a continuous flow of $\text{N}_2:\text{CO}_2$ gas mixture (80:20). After the transfer of medium aliquots in the serum bottles, the headspace was replaced by a $\text{H}_2:\text{CO}_2$ gas mixture (80:20). The measurements for the assessment of methane production were made at the premises of IMBBC, Heraklion Crete using a gas chromatography system equipped with a flame ionization detector and a thermal conductivity detector in series (GC-FID/TCD).

DNA extraction, PCR and cloning

The enrichment culture showing the highest methanogenic activity was selected for the extraction/isolation of genetic material and the amplification of a highly conserved ribosomal RNA gene, which is typically used as a marker for the identification of microbial species. Approximately 2 ml of microbial culture were used for the extraction of genetic material using the DNA MOBIO Fast Spin kit for Soil (MOBIO, USA) and following the instructions of the manufacturer. In order to amplify bacterial 16S rRNA genes, the technique of polymerase chain reaction (PCR) was applied using the bacterial primers 27f and 1492r, and by following the procedure described in a previous study of Polymenakou *et al.* (2005). In brief, 6 separate reactions of 20 μl each were prepared and subjected to polymerase chain reaction using a thermocycler and the following temperature program: initial denaturation step at 94°C for 3 min followed by 30 thermal cycles each consisting of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C and a final elongation stage at 94°C for 7 min. Each reaction contained the following ingredients: 1–4 ng of microbial DNA extract, PCR buffer [10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, and 2 mM MgCl_2], 100 nM of each primer, 200 mM of each deoxyribonucleotide triphosphate and 0.25 U of Taq DNA polymerase enzyme (Invitrogen, Carlsbad, CA, USA). The products of the six reactions were pooled together and purified using appropriate protocols in order to remove interfering substances and enable cloning into the pCR 4-TOPO vector. The final cloned product was transferred into chemically competent cells of *Escherichia coli* (One shot TOP10) using the TOPO TA Cloning kit (Version M) of Invitrogen. The same procedure was applied for the amplification/cloning of the archaeal 16S rRNA genes using the primers 8f and 927r. A total of 96 bacterial and archaeal clones were collected and grown into Luria-Bertani medium containing kanamycin at 50 mg/ml. Cells were subsequently lysed and subjected to sequencing.

Sequencing and phylogenetic analysis

The sequencing reactions were undertaken using the Bac-27f primer for bacterial clones (Lane, 1991) and Arch-8f primer for archaeal clones (Teske *et al.*, 2002), following the protocol provided by the BigDye terminator kit v3.1 from Applied Biosystems. DNA sequencing was performed on a ABI 3730 capillary sequencer (Applied Biosystems), which is available at the laboratories of IMBBC, Heraklion Crete. This generated high-quality read of between 660 and 870 bases. The obtained sequences were compared against the 16S rRNA gene sequences deposited in the GenBank database in order to ascertain the closest relatives of bacteria/archaea species present in the most active sample. 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 firstly aligned by ClustalW (Version 2.1) (Larkin *et al.*, 2007) using gap opening penalty 7, gap extension penalty 2 for both pairwise as well as multiple alignments, DNA weight matrix IUB and transition weight 0.1. Aligned sequences were then subjected to phylogenetic analysis employing Bayesian statistics via MrBayes (Version 3.2.6) (Ronquist *et al.*, 2003) using following parameters: mixed model of nucleotide substitution, gamma-distributed rates among sites, four Monte Carlo Markov chains for 2 000 000 cycles, chains were sampled every 1000th generation, first 25% of the samples were discarded as burn-in and *Methanoculleus sp. SLH121* was used as outgroup. 50% majority-rule consensus was applied in order to generate final tree topology. Resulting tree topology was visualized via iTOL (Version 3.5.4) (Letunic & Bork, 2016) and edited using Inkscape (Version 0.91) (www.inkscape.org).

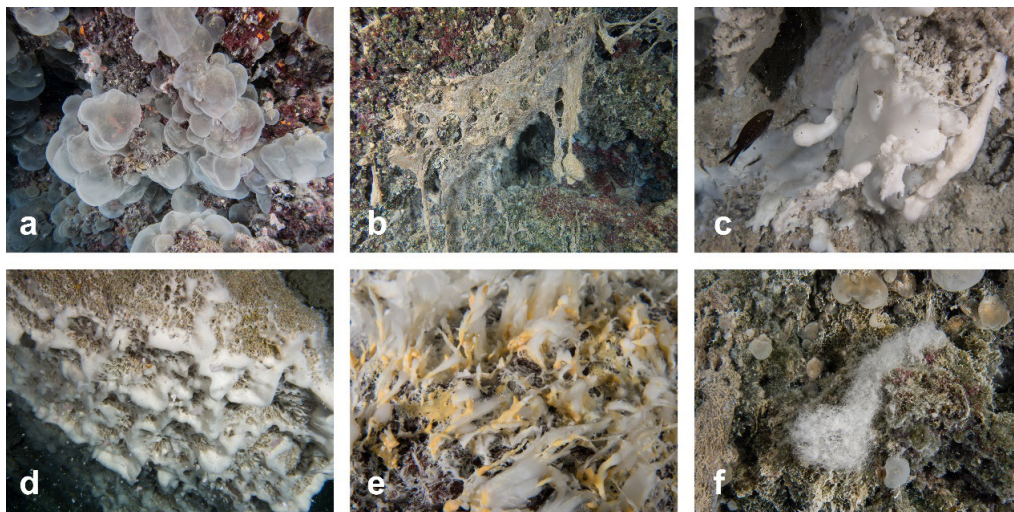


Fig. S1: Underwater images of the six different types of microbial colonies from Zakynthos submarine caves that were collected and used for the implementation of cultivation experiments. Samples No 4 (a) and 1 (b) were egg-shaped colonies, sample No 6 (c) and No 3 (d) were foam-like colonies whereas samples No 5 (e), 8 and 14 (f) were filamentous microbial mats.



Fig. S2: Transfer of the culture medium into serum bottles under anaerobic conditions and preparation of multiple cultures for the investigation of methane production.

Table S1. Concentration data (% v/v) obtained from consecutive measurements of methane in the headspace of fourteen anaerobic cultures (2 blanks and 2 replicates for each of the six different cave samples i.e. codes 1, 3, 4, 5, 6, 8/14) over a period of 208 days. The cultures showing strong methanogenicity are highlighted in red fonts. Culture No 1A was used for microbial community analysis. Samples No1 and 4 were egg-shaped colonies and 3 was a foam-like colony.

Days -->	16	40	58	64	79	80	95	131	154	165	172	194	208
BL1	0.004	0.004	0	0	0.001	-	-	-	-	-	-	-	-
BL2	0.001	0.001	0.001	0.001	0	-	-	0	0.001	0.001	0	-	-
No1A	0.001	8.122	for 16S	-	-	-	-	-	-	-	-	-	-
No1B	0.003	0.001	5.861	7.304	8.478	-	-	7.552	1.926	4.15	1.835	17.43	8.599
No3A	0.001	0.001	0.014	0.032	6.319	-	-	8.373	12.4	9.838	2.065	21.03	8.71
No3B	0.001	0.001	0.013	0.015	0.017	-	0.017	0.057	0.011	0.006	-	-	-
No4A	0.001	0.001	0.014	0.014	2.725	3.029	2.45	2.316	29.8	11.48	0.172	0.859	1.741
No4B	0.001	0.001	0.014	-	0.014	0.022	0.016	0.027	0.007	0.006	-	-	-
No5A	0.001	0.001	-	-	-	-	-	-	-	-	-	-	-
No5B	0.001	0.001	0.01	0.013	0.013	-	-	0.034	-	-	-	-	-
No6A	0.001	0.001	0.011	0.015	-	0.014	-	0.037	-	-	-	-	-
No6B	0.001	0.001	0.01	-	-	0.012	-	0.033	-	-	-	-	-
No8A	0.001	0.001	0.011	0.015	-	0.016	0.016	0.028	-	-	-	-	-
No14B	0.001	0.001	0.009	-	-	0.011	-	0.036	-	-	-	-	-

Table S2. List of all sequenced enrichment culture clones, OTUs, family-level affiliation and their GenBank accession numbers. Identical sequences (i.e. sequences showing similarity of 100%) were grouped into unique operational taxonomic units (OTUs) and were indicated as Bacterial OTU A-K and Archaeal OTU A-C. For the rest of them, which were represented only once in the dataset, we used the same name with the sequenced clone.

<i>Sequenced clone</i>	<i>OTU</i>	<i>Family</i>	<i>Accession numbers</i>
CavesP11_5B_7f	Archaeal OTU A	Methanomicrobiaceae	MF627366
CavesP11_3E_7f	Archaeal OTU B	Methanomicrobiaceae	MF627333
CavesP11_3F_7f	Archaeal OTU B	Methanomicrobiaceae	MF627334
CavesP11_3H_7f	Archaeal OTU B	Methanomicrobiaceae	MF627349
CavesP11_4D_7f	Archaeal OTU B	Methanomicrobiaceae	MF627361
CavesP11_5C_7f	Archaeal OTU B	Methanomicrobiaceae	MF627370
CavesP11_4E_7f	Archaeal OTU B	Methanomicrobiaceae	MF627376
CavesP11_4F_7f	Archaeal OTU B	Methanomicrobiaceae	MF627377
CavesP11_4B_7f	Archaeal OTU C	Methanomicrobiaceae	MF627350
CavesP11_4C_7f	Archaeal OTU C	Methanomicrobiaceae	MF627360
CavesP12_2C_27f	Bacterial OTU H	Desulfobulbaceae	MF627330
CavesP12_9F_27f	Bacterial OTU H	Desulfobulbaceae	MF627382
CavesP12_9E_27f	Bacterial OTU H	Desulfobulbaceae	MF627384
CavesP12_10D_27f	Bacterial OTU H	Desulfobulbaceae	MF627387
CavesP12_10C_27f	Bacterial OTU H	Desulfobulbaceae	MF627389
CavesP12_12G_27f	Bacterial OTU H	Desulfobulbaceae	MF627399
CavesP12_3F_27f	Bacterial OTU I	Desulfobulbaceae	MF627339
CavesP12_4F_27f	Bacterial OTU I	Desulfobulbaceae	MF627340
CavesP12_5D_27f	Bacterial OTU I	Desulfobulbaceae	MF627403
CavesP12_11D_27f	Bacterial OTU I	Desulfobulbaceae	MF627407
CavesP12_12F_27f	Bacterial OTU J	Desulfobulbaceae	MF627402
CavesP12_11E_27f	Bacterial OTU J	Desulfobulbaceae	MF627404
CavesP12_8H_27f	Bacterial OTU K	Desulfobulbaceae	MF627363
CavesP12_9B_27f	Bacterial OTU K	Desulfobulbaceae	MF627390
CavesP12_12D_27f	Bacterial OTU K	Desulfobulbaceae	MF627408
CavesP12_1B_27f	CavesP12_1B_27f	Desulfobulbaceae	MF627331

(continued)

Table S2 continued

<i>Sequenced clone</i>	<i>OTU</i>	<i>Family</i>	<i>Accession numbers</i>
CavesPI2_4D_27f	CavesPI2_4D_27f	Desulfobulbaceae	MF627344
CavesPI2_3B_27f	CavesPI2_3B_27f	Desulfobulbaceae	MF627347
CavesPI2_3H_27f	CavesPI2_3H_27f	Desulfobulbaceae	MF627335
CavesPI2_7G_27f	CavesPI2_7G_27f	Desulfobulbaceae	MF627364
CavesPI2_7B_27f	CavesPI2_7B_27f	Desulfobulbaceae	MF627374
CavesPI2_8B_27f	CavesPI2_8B_27f	Desulfobulbaceae	MF627375
CavesPI2_9H_27f	CavesPI2_9H_27f	Desulfobulbaceae	MF627378
CavesPI2_9G_27f	CavesPI2_9G_27f	Desulfobulbaceae	MF627380
CavesPI2_10G_27f	CavesPI2_10G_27f	Desulfobulbaceae	MF627381
CavesPI2_10F_27f	CavesPI2_10F_27f	Desulfobulbaceae	MF627383
CavesPI2_9C_27f	CavesPI2_9C_27f	Desulfobulbaceae	MF627388
CavesPI2_9A_27f	CavesPI2_9A_27f	Desulfobulbaceae	MF627392
CavesPI2_6D_27f	CavesPI2_6D_27f	Desulfobulbaceae	MF627397
CavesPI2_3E_27f	Bacterial OTU E	Desulfuromonaceae	MF627341
CavesPI2_4E_27f	Bacterial OTU E	Desulfuromonaceae	MF627342
CavesPI2_4C_27f	Bacterial OTU E	Desulfuromonaceae	MF627346
CavesPI2_4B_27f	Bacterial OTU E	Desulfuromonaceae	MF627348
CavesPI2_5G_27f	Bacterial OTU E	Desulfuromonaceae	MF627353
CavesPI2_6G_27f	Bacterial OTU E	Desulfuromonaceae	MF627354
CavesPI2_6F_27f	Bacterial OTU E	Desulfuromonaceae	MF627356
CavesPI2_6C_27f	Bacterial OTU E	Desulfuromonaceae	MF627357
CavesPI2_7H_27f	Bacterial OTU E	Desulfuromonaceae	MF627362
CavesPI2_8E_27f	Bacterial OTU E	Desulfuromonaceae	MF627369
CavesPI2_8C_27f	Bacterial OTU E	Desulfuromonaceae	MF627373
CavesPI2_11H_27f	Bacterial OTU E	Desulfuromonaceae	MF627395
CavesPI2_12H_27f	Bacterial OTU E	Desulfuromonaceae	MF627396
CavesPI2_4E_27f2	Bacterial OTU E	Desulfuromonaceae	MF627414
CavesPI2_11A_27f	Bacterial OTU E	Desulfuromonaceae	MF627415
CavesPI2_1H_27f	Bacterial OTU F	Desulfuromonaceae	MF627323
CavesPI2_8G_27f	Bacterial OTU F	Desulfuromonaceae	MF627365
CavesPI2_1C_27f	CavesPI2_1C_27f	Desulfuromonaceae	MF627329
CavesPI2_4H_27f	CavesPI2_4H_27f	Desulfuromonaceae	MF627336
CavesPI2_3D_27f	CavesPI2_3D_27f	Desulfuromonaceae	MF627343
CavesPI2_5F_27f	CavesPI2_5F_27f	Desulfuromonaceae	MF627355
CavesPI2_5B_27f	CavesPI2_5B_27f	Desulfuromonaceae	MF627358
CavesPI2_8F_27f	CavesPI2_8F_27f	Desulfuromonaceae	MF627367
CavesPI2_10B_27f	CavesPI2_10B_27f	Desulfuromonaceae	MF627391
CavesPI2_5E_27f	CavesPI2_5E_27f	Desulfuromonaceae	MF627400
CavesPI2_2E_27f	CavesPI2_2E_27f	Desulfuromonaceae	MF627409
CavesPI2_11C_27f	CavesPI2_11C_27f	Desulfuromonaceae	MF627410
CavesPI2_11B_27f	CavesPI2_11B_27f	Desulfuromonaceae	MF627413
CavesPI2_7C_27f	Bacterial OTU A	Clostridiaceae	MF627372
CavesPI2_12E_27f	Bacterial OTU A	Clostridiaceae	MF627405
CavesPI2_3C_27f	CavesPI2_3C_27f	Clostridiaceae	MF627345
CavesPI2_3G_27f	Bacterial OTU G	Desulfobacteraceae	MF627337
CavesPI2_2G_27f	Bacterial OTU G	Desulfobacteraceae	MF627406
CavesPI2_11G_27f	CavesPI2_11G_27f	Desulfobacteraceae	MF627398
CavesPI2_10H_27f	CavesPI2_10H_27f	Marinifilaceae	MF627379

(continued)

Table S2 continued

<i>Sequenced clone</i>	<i>OTU</i>	<i>Family</i>	<i>Accession numbers</i>
CavesPI2_9D_27f	CavesPI2_9D_27f	Marinifilaceae	MF627386
CavesPI2_2B_27f	CavesPI2_2B_27f	Campylobacteraceae	MF627332
CavesPI2_2H_27f	CavesPI2_2H_27f	Incertae Sedis - Family I	MF627324
CavesPI2_2D_27f	Bacterial OTU B	Incertae Sedis - Family II	MF627328
CavesPI2_10A_27f	Bacterial OTU B	Incertae Sedis - Family II	MF627393
CavesPI2_7D_27f	Bacterial OTU C	Incertae Sedis - Family II	MF627371
CavesPI2_6E_27f	Bacterial OTU C	Incertae Sedis - Family II	MF627394
CavesPI2_6B_27f	Bacterial OTU D	Incertae Sedis - Family II	MF627359
CavesPI2_10E_27f	Bacterial OTU D	Incertae Sedis - Family II	MF627385
CavesPI2_4G_27f2	Bacterial OTU D	Incertae Sedis - Family II	MF627412
CavesPI2_1F_27f	CavesPI2_1F_27f	Incertae Sedis - Family II	MF627325
CavesPI2_2F_27f	CavesPI2_2F_27f	Incertae Sedis - Family II	MF627326
CavesPI2_1D_27f	CavesPI2_1D_27f	Incertae Sedis - Family II	MF627327
CavesPI2_4G_27f	CavesPI2_4G_27f	Incertae Sedis - Family II	MF627338
CavesPI2_5H_27f	CavesPI2_5H_27f	Incertae Sedis - Family II	MF627351
CavesPI2_7E_27f	CavesPI2_7E_27f	Incertae Sedis - Family II	MF627368
CavesPI2_11F_27f	CavesPI2_11F_27f	Incertae Sedis - Family II	MF627401

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