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

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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; $MgCl_2 \cdot 6H_2O$, 0.16; $CaCl_2 \cdot 2H_2O$, 0.009; resazurin 0.1% solution, 1 ml; cysteine · HCl 200mM, 15ml; $Na_2S \cdot 9H_2O$ 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, a-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; $Fe(NH_4)_2(SO_4)_2$, 0.8; $NaSeO_3$, 0.2; $CoCl_2 \cdot 6H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.1; $NaWO_4 \cdot 2H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.1; H_3BO_3, 0.01; $CuSO_4 \cdot 5H_2O$, 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 N_2:CO_2 gas mixture (80:20). After the transfer of medium aliquots in the serum bottles, the headspace was replaced by a H_2: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 lyzed 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).

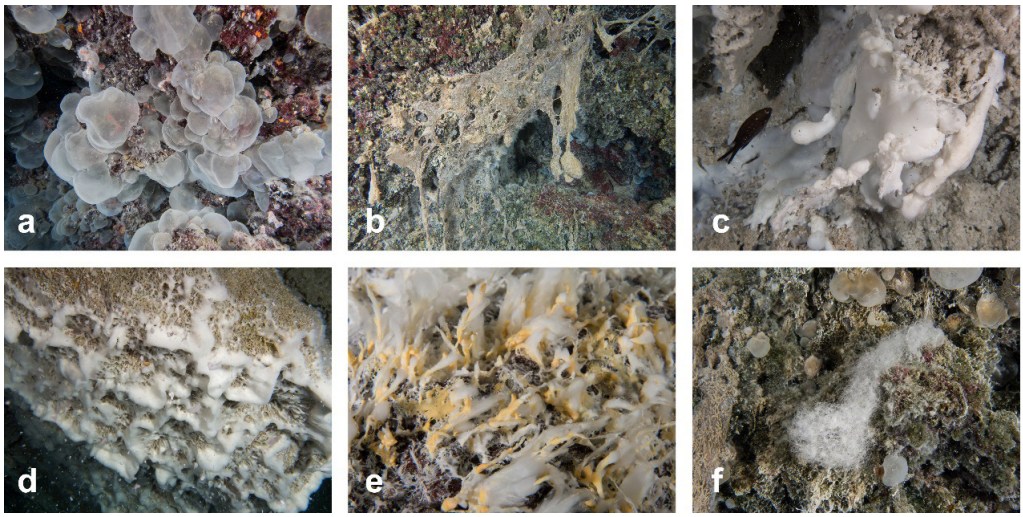

Fig. S1: Underwater images of the six different types of microbial colonies from Zakynthos submarine caves that were collected and used for the implementation of cultivation experiments. Samples No 4 (a) and 1 (b) were egg-shaped colonies, sample No 6 (c) and No 3 (d) were foam-like colonies whereas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

Sequenced clone	OTU	Family	Accession numbers
CavesPl1_5B_7f	Archaeal OTU A	Methanomicrobiaceae	MF627366
CavesPl1_3E_7f	Archaeal OTU B	Methanomicrobiaceae	MF627333
CavesPl1_3F_7f	Archaeal OTU B	Methanomicrobiaceae	MF627334
CavesPl1_3H_7f	Archaeal OTU B	Methanomicrobiaceae	MF627349
CavesPl1_4D_7f	Archaeal OTU B	Methanomicrobiaceae	MF627361
CavesPl1_5C_7f	Archaeal OTU B	Methanomicrobiaceae	MF627370
CavesPl1_4E_7f	Archaeal OTU B	Methanomicrobiaceae	MF627376
CavesPl1_4F_7f	Archaeal OTU B	Methanomicrobiaceae	MF627377
CavesPl1_4B_7f	Archaeal OTU C	Methanomicrobiaceae	MF627350
CavesPl1_4C_7f	Archaeal OTU C	Methanomicrobiaceae	MF627360
CavesPl2_2C_27f	Bacterial OTU H	Desulfobulbaceae	MF627330
CavesPl2_9F_27f	Bacterial OTU H	Desulfobulbaceae	MF627382
CavesPl2_9E_27f	Bacterial OTU H	Desulfobulbaceae	MF627384
CavesPl2_10D_27f	Bacterial OTU H	Desulfobulbaceae	MF627387
CavesPl2_10C_27f	Bacterial OTU H	Desulfobulbaceae	MF627389
CavesPl2_12G_27f	Bacterial OTU H	Desulfobulbaceae	MF627399
CavesPl2_3F_27f	Bacterial OTU I	Desulfobulbaceae	MF627339
CavesPl2_4F_27f	Bacterial OTU I	Desulfobulbaceae	MF627340
CavesPl2_5D_27f	Bacterial OTU I	Desulfobulbaceae	MF627403
CavesPl2_11D_27f	Bacterial OTU I	Desulfobulbaceae	MF627407
CavesPl2_12F_27f	Bacterial OTU J	Desulfobulbaceae	MF627402
CavesPl2_11E_27f	Bacterial OTU J	Desulfobulbaceae	MF627404
CavesPl2_8H_27f	Bacterial OTU K	Desulfobulbaceae	MF627363
CavesPl2_9B_27f	Bacterial OTU K	Desulfobulbaceae	MF627390
CavesPl2_12D_27f	Bacterial OTU K	Desulfobulbaceae	MF627408
CavesPl2_1B_27f	CavesPl2_1B_27f	Desulfobulbaceae	MF627331

(continued)

Table S2 continued

Sequenced clone	*OTU*	*Family*	*Accession numbers*
CavesPl2_4D_27f	CavesPl2_4D_27f	Desulfobulbaceae	MF627344
CavesPl2_3B_27f	CavesPl2_3B_27f	Desulfobulbaceae	MF627347
CavesPl2_3H_27f	CavesPl2_3H_27f	Desulfobulbaceae	MF627335
CavesPl2_7G_27f	CavesPl2_7G_27f	Desulfobulbaceae	MF627364
CavesPl2_7B_27f	CavesPl2_7B_27f	Desulfobulbaceae	MF627374
CavesPl2_8B_27f	CavesPl2_8B_27f	Desulfobulbaceae	MF627375
CavesPl2_9H_27f	CavesPl2_9H_27f	Desulfobulbaceae	MF627378
CavesPl2_9G_27f	CavesPl2_9G_27f	Desulfobulbaceae	MF627380
CavesPl2_10G_27f	CavesPl2_10G_27f	Desulfobulbaceae	MF627381
CavesPl2_10F_27f	CavesPl2_10F_27f	Desulfobulbaceae	MF627383
CavesPl2_9C_27f	CavesPl2_9C_27f	Desulfobulbaceae	MF627388
CavesPl2_9A_27f	CavesPl2_9A_27f	Desulfobulbaceae	MF627392
CavesPl2_6D_27f	CavesPl2_6D_27f	Desulfobulbaceae	MF627397
CavesPl2_3E_27f	Bacterial OTU E	Desulfuromonaceae	MF627341
CavesPl2_4E_27f	Bacterial OTU E	Desulfuromonaceae	MF627342
CavesPl2_4C_27f	Bacterial OTU E	Desulfuromonaceae	MF627346
CavesPl2_4B_27f	Bacterial OTU E	Desulfuromonaceae	MF627348
CavesPl2_5G_27f	Bacterial OTU E	Desulfuromonaceae	MF627353
CavesPl2_6G_27f	Bacterial OTU E	Desulfuromonaceae	MF627354
CavesPl2_6F_27f	Bacterial OTU E	Desulfuromonaceae	MF627356
CavesPl2_6C_27f	Bacterial OTU E	Desulfuromonaceae	MF627357
CavesPl2_7H_27f	Bacterial OTU E	Desulfuromonaceae	MF627362
CavesPl2_8E_27f	Bacterial OTU E	Desulfuromonaceae	MF627369
CavesPl2_8C_27f	Bacterial OTU E	Desulfuromonaceae	MF627373
CavesPl2_11H_27f	Bacterial OTU E	Desulfuromonaceae	MF627395
CavesPl2_12H_27f	Bacterial OTU E	Desulfuromonaceae	MF627396
CavesPl2_4E_27f2	Bacterial OTU E	Desulfuromonaceae	MF627414
CavesPl2_11A_27f	Bacterial OTU E	Desulfuromonaceae	MF627415
CavesPl2_1H_27f	Bacterial OTU F	Desulfuromonaceae	MF627323
CavesPl2_8G_27f	Bacterial OTU F	Desulfuromonaceae	MF627365
CavesPl2_1C_27f	CavesPl2_1C_27f	Desulfuromonaceae	MF627329
CavesPl2_4H_27f	CavesPl2_4H_27f	Desulfuromonaceae	MF627336
CavesPl2_3D_27f	CavesPl2_3D_27f	Desulfuromonaceae	MF627343
CavesPl2_5F_27f	CavesPl2_5F_27f	Desulfuromonaceae	MF627355
CavesPl2_5B_27f	CavesPl2_5B_27f	Desulfuromonaceae	MF627358
CavesPl2_8F_27f	CavesPl2_8F_27f	Desulfuromonaceae	MF627367
CavesPl2_10B_27f	CavesPl2_10B_27f	Desulfuromonaceae	MF627391
CavesPl2_5E_27f	CavesPl2_5E_27f	Desulfuromonaceae	MF627400
CavesPl2_2E_27f	CavesPl2_2E_27f	Desulfuromonaceae	MF627409
CavesPl2_11C_27f	CavesPl2_11C_27f	Desulfuromonaceae	MF627410
CavesPl2_11B_27f	CavesPl2_11B_27f	Desulfuromonaceae	MF627413
CavesPl2_7C_27f	Bacterial OTU A	Clostridiaceae	MF627372
CavesPl2_12E_27f	Bacterial OTU A	Clostridiaceae	MF627405
CavesPl2_3C_27f	CavesPl2_3C_27f	Clostridiaceae	MF627345
CavesPl2_3G_27f	Bacterial OTU G	Desulfobacteraceae	MF627337
CavesPl2_2G_27f	Bacterial OTU G	Desulfobacteraceae	MF627406
CavesPl2_11G_27f	CavesPl2_11G_27f	Desulfobacteraceae	MF627398
CavesPl2_10H_27f	CavesPl2_10H_27f	Marinifilaceae	MF627379

(continued)

Table S2 continued

Sequenced clone	*OTU*	*Family*	*Accession numbers*
CavesPl2_9D_27f	CavesPl2_9D_27f	Marinifilaceae	MF627386
CavesPl2_2B_27f	CavesPl2_2B_27f	Campylobacteraceae	MF627332
CavesPl2_2H_27f	CavesPl2_2H_27f	Incertae Sedis - Familly I	MF627324
CavesPl2_2D_27f	Bacterial OTU B	Incertae Sedis - Familly II	MF627328
CavesPl2_10A_27f	Bacterial OTU B	Incertae Sedis - Familly II	MF627393
CavesPl2_7D_27f	Bacterial OTU C	Incertae Sedis - Familly II	MF627371
CavesPl2_6E_27f	Bacterial OTU C	Incertae Sedis - Familly II	MF627394
CavesPl2_6B_27f	Bacterial OTU D	Incertae Sedis - Familly II	MF627359
CavesPl2_10E_27f	Bacterial OTU D	Incertae Sedis - Familly II	MF627385
CavesPl2_4G_27f2	Bacterial OTU D	Incertae Sedis - Familly II	MF627412
CavesPl2_1F_27f	CavesPl2_1F_27f	Incertae Sedis - Familly II	MF627325
CavesPl2_2F_27f	CavesPl2_2F_27f	Incertae Sedis - Familly II	MF627326
CavesPl2_1D_27f	CavesPl2_1D_27f	Incertae Sedis - Familly II	MF627327
CavesPl2_4G_27f	CavesPl2_4G_27f	Incertae Sedis - Familly II	MF627338
CavesPl2_5H_27f	CavesPl2_5H_27f	Incertae Sedis - Familly II	MF627351
CavesPl2_7E_27f	CavesPl2_7E_27f	Incertae Sedis - Familly II	MF627368
CavesPl2_11F_27f	CavesPl2_11F_27f	Incertae Sedis - Familly II	MF627401

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