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): NH₄Cl, 1; NaCl, 0.6; NaHCO₃, 5; KH₂PO₄, 0.3; KH₂PO₄, 0.3; MgCl₂ • 6H₂O, 0.16; CaCl₂ • 2H₂O, 0.009; resazurin 0.1% solution, 1 ml; cysteine • HCl 200mM, 15ml; Na,S • 9H,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, 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(NH₄)₂(SO₄)_2, 0.8; NaSeO₃, 0.2; CoCl₂ • 6H₂O, 0.1; MnSO₄ • H₂O, 0.1; Na₂MoO₄ • 2H₂O, 0.1; NaWO₄ • 2H₂O, 0.1; ZnSO₄ • 7H₂O, 0.1; NiCl₂ • 6H₂O, 0.1; H₃BO₃, 0.01; CuSO₄ • 5H₂O, 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 N₂:CO₂ gas mixture (80:20). After the transfer of medium aliquots in the serum bottles, the headspace was replaced by a H₂:CO₂ 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 MgCl₂], 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 | 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 | 0.014 | 0.014 | 0.022 | 0.016 | 0.027 | 0.007 | 0.006 | - | - | - |
| No5A   | 0.001 | 0.001 | - | - | - | - | - | - | - | - | - | - | - |
| No5B   | 0.001 | 0.001 | 0.013 | 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)
| 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 | MF627392 |
| CavesPl2_6D_27f | CavesPl2_6D_27f | Desulfobulbaceae | MF627397 |
| CavesPl2_3E_27f | CavesPl2_3E_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_4E_27f | CavesPl2_4E_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_4C_27f | CavesPl2_4C_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_4B_27f | CavesPl2_4B_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_5G_27f | CavesPl2_5G_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_6G_27f | CavesPl2_6G_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_6F_27f | CavesPl2_6F_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_6C_27f | CavesPl2_6C_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_7H_27f | CavesPl2_7H_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_8E_27f | CavesPl2_8E_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_8C_27f | CavesPl2_8C_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_11H_27f| CavesPl2_11H_27f| Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_12H_27f| CavesPl2_12H_27f| Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_4E_27f| CavesPl2_4E_27f | Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_11A_27f| CavesPl2_11A_27f| Bacterial OTU E | Desulfuromonaceae |
| CavesPl2_1H_27f | CavesPl2_1H_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_8G_27f | CavesPl2_8G_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_1C_27f | CavesPl2_1C_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_4H_27f | CavesPl2_4H_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_3D_27f | CavesPl2_3D_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_5F_27f | CavesPl2_5F_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_5B_27f | CavesPl2_5B_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_8F_27f | CavesPl2_8F_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_10B_27f| CavesPl2_10B_27f| Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_5E_27f | CavesPl2_5E_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_2E_27f | CavesPl2_2E_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_11C_27f| CavesPl2_11C_27f| Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_11B_27f| CavesPl2_11B_27f| Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_7C_27f | CavesPl2_7C_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_12E_27f| CavesPl2_12E_27f| Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_3C_27f | CavesPl2_3C_27f | Bacterial OTU F | Desulfuromonaceae |
| CavesPl2_3G_27f | CavesPl2_3G_27f | Bacterial OTU G | Desulfobacteraceae |
| CavesPl2_2G_27f | CavesPl2_2G_27f | Bacterial OTU G | Desulfobacteraceae |
| CavesPl2_11G_27f| CavesPl2_11G_27f| Bacterial OTU G | Desulfobacteraceae |
| CavesPl2_10H_27f| CavesPl2_10H_27f| Bacterial OTU G | Desulfobacteraceae |

(continued)
Table S2 continued

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

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